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## FURTHER EXPERIENCES WITH STREPTOMYCIN THERAPY IN UNITED STATES ARMY HOSPITALS

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**E**VALUATION of streptomycin was begun in United States Army Hospitals in 1945. This investigation is still in progress. A special research unit established originally at Halloran General Hospital is now continuing to function at Brooke General Hospital in San Antonio. Here sensitivities of bacteria to streptomycin have been tested and the findings correlated with studies on absorption, distribution, and excretion of the drug in the body following parenteral administration.<sup>1</sup> This unit in addition is carrying on intensive studies on certain types of infections, especially surgical. Data on these and on all patients treated with streptomycin in U. S. Army Hospitals are recorded on special forms for permanent retention. It is our purpose here to present in summary form these cumulative experiences based on an analysis of 1,200 cases.

A complete understanding of the properties of streptomycin is an absolute necessity if the drug is to be used intelligently. The principal characteristics<sup>1,2</sup> are as follows: (1) Streptomycin is an organic base freely soluble in water but not in the common organic solvents. (2) Solutions of this compound are remarkably stable both chemically and biologically. Refrigeration is optional for short periods of storage. (3) In contrast to penicillin streptomycin is not destroyed by enzymes or by bacteria. (4) The drug is standardized so that 1 S. unit is approximately equivalent to 1 microgram. (5) A wide variety of pathogenic gram-negative and gram-positive bacteria are susceptible in vitro to streptomycin (Table I). Streptomycin is highly selective in its antibacterial activity, different strains showing marked variation in their sensitivity to the drug, and naturally drug-fast strains are encountered in nearly all species. (6) The activity of the drug is depressed by low pH, dextrose, high concentrations of inorganic salts and reducing substances. (7) Clinical response to this antibiotic is closely related to the in vitro sensitivity of the bacteria. Infections caused by organisms with high in vitro sensitivities may respond favorably, whereas those with low sensitivities are usually refractory to treatment. (8) Absorption, excretion and distribution of streptomycin generally follow the same pattern after parenteral administration as penicillin. The exceptions are that no absorption into the general circulation takes place following administration orally or by nebulization. (9) Bacteria acquire fastness to

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*monas aeruginosa* and *Streptococcus faecalis* There was no appreciable difference in results of therapy between monobacterial and polybacterial infections provided that all organisms were susceptible in vitro to streptomycin The marked constitutional signs and symptoms accompanying acute pyelonephritis or acute exacerbations of chronic pyelonephritis responded dramatically to streptomycin, mild urinary tract symptoms and low grade fever responded more irregularly Patients with paraplegia were benefited by streptomycin therapy, although the effects were rarely permanent except in patients with closed urinary systems and automatic bladders In these patients clinical remissions were not infrequent in spite of the fact that complete sterility of the urine was not achieved Presence of obstructive calculi precluded a successful chemotherapeutic result Advanced renal pathology, undrained abscesses, presence of neoplasms, and infections in the prostate were other causes of failure of streptomycin therapy Drug fastness is a constant feature in cases in which the prescribed course of therapy has not sterilized the urine No advantage accrued from the administration of a second course of the drug if the first course had failed to sterilize the urine Local instillations of streptomycin into the urinary tract via urethral catheters, cystostomy or pyelostomy tubes have been found to be of no value in our experience<sup>7</sup>

A dosage of 12 to 24 Gm per day of streptomycin in three or four hourly divided doses for three to five days is recommended for treatment of the average infection of the urinary tract due to susceptible organisms Alkalinization of the urine with streptomycin therapy may be advantageous in selected cases There is no contraindication to the concurrent use of penicillin or sulfonamides with streptomycin if warranted by the clinical and laboratory findings The percentage of satisfactory results is highest when the requirements of (1) free flow of urine, (2) susceptible organisms and (3) adequate dosage of the drug are met

Streptomycin may be a useful agent in uncomplicated nongonococcal urethritis of bacterial origin and in acute epididymitis when the organisms are susceptible<sup>8</sup> Uncomplicated gonorrheal urethritis responds to streptomycin as dramatically as to penicillin, except when there is prostatic involvement In tubovarian disease, particularly disease of less than one year's duration the antibiotic seems of value in those instances in which penicillin and the sulfonamides no longer are effective The drug has a place in the management of sinus tracts of urinary tract origin infected with streptomycin sensitive organisms but will not eliminate surgery in most instances In patients with Reiter's syndrome and amicrobial cystitis of unknown etiology, no consistently beneficial effects have been observed with streptomycin therapy Neither parenteral nor oral administration can produce an effective concentration of streptomycin in prostatic secretions, which explains why this compound has not proved beneficial in the treatment of prostatitis<sup>9</sup>

*Typhemia*—Ten patients with typhemia were treated with streptomycin one with the oculoglandular type one with the typhoidal type, and the others

streptomycin more rapidly than to penicillin (10) This drug is relatively nontoxic when given for short periods of time Toxicity is commonly observed when the courses of therapy are prolonged

TABLE I BACTERIA SUSCEPTIBLE IN VITRO TO STREPTOMYCIN

<i>Aerobacter aerogenes</i>	<i>Pasteurella tularensis</i>
<i>Alkaligenes faecalis</i>	<i>Proteus morganii</i>
<i>Brucella abortus</i>	<i>Proteus vulgaris</i>
<i>Brucella melitensis</i>	<i>Pseudomonas aeruginosa</i>
<i>Eberthella typhosa</i>	<i>Salmonella</i> species
<i>Escherichia coli</i>	<i>Shigella</i> species
<i>Hemophilus ducreyi</i>	<i>Streptobacillus moniliformis</i>
<i>Hemophilus influenzae</i>	<i>Actinomyces bovis</i> *
<i>Hemophilus pertussis</i>	<i>Bacillus anthracis</i> *
<i>Klebsiella ozaenae</i>	<i>Corynebacterium diphtheriae</i> *
<i>Klebsiella pneumoniae</i>	<i>Diplococcus pneumoniae</i> *
<i>Neisseria gonorrhoeae</i> *	<i>Mycobacterium tuberculosis</i>
<i>Neisseria intracellularis</i> *	<i>Staphylococci</i> *
<i>Pasteurella pestis</i>	<i>Streptococci, hemolytic</i> *
	<i>Streptococci, nonhemolytic</i>

Streptomycin is ineffective clinically against anaerobic mycotic protozoan viral and spirochetal infections and worm infestations Rickettsiae of epidemic and murine typhus Rocky Mountain spotted fever and rickettsialpox are sensitive in vitro to streptomycin

\*Penicillin-sensitive

#### RESULTS OF THERAPY

The following are illustrative of types of infections which, in our experience, have shown various degrees of response to streptomycin

*Infections of the Urogenital Tract*—Four hundred and sixty-five patients with infection of the urinary tract were submitted to critical analysis and study<sup>12</sup> All patients had a complete bacteriologic survey of the urine prior to treatment Culture sensitivity tests revealed that streptomycin had a bacteriostatic effect in vitro on 87 per cent of the bacteria recovered before treatment, while the remaining 13 per cent were naturally drug-fast Seventy-nine per cent of the microorganisms isolated were gram-negative bacilli The remaining 21 per cent were gram-positive cocci, a third of these nonhemolytic streptococci The survey showed that if bacteria are not inhibited in vitro by a concentration of 16  $\mu$ g per cubic centimeter, the chances of eliminating them by streptomycin therapy are not good Streptomycin intramuscularly administered can maintain a concentration in excess of 16  $\mu$ g per milliliter of blood serum The drug is excreted in very high concentrations in the urine (1,000  $\mu$ g per milliliter or more) This suggests that streptomycin acts primarily through delivery by way of the blood stream to the tissues and not through the urine

Of the four hundred and sixty-five patients treated, an outright cure was obtained in 34 per cent of the patients, an additional 21 per cent were improved, that is symptoms were ameliorated, but without complete bacteriologic remission The remaining 45 per cent were not benefited As experience was gained concerning the factors which limit successful streptomycin therapy in urinary tract infections, proper selection of cases led to a progressively higher rate of cures Best results were obtained when infections were caused by *Escherichia coli* Failures were especially frequent when the causal organisms were *Pseudo*

In nearly every instance temperature subsided by a stepladder type of lysis. In most instances the blood was rapidly sterilized of the bacteria, with surgical drainage contributing to the most rapid clearing of the blood stream. Clinical improvement was usually evident by the third or fourth day of treatment. With urinary sepsis the bacteremias cleared rapidly, but pyuria continued until the foci of infection were removed. In cases of bone lesions the infection was localized by streptomycin, but surgical intervention was necessary in the management of the primary focus.

Laboratory studies show that an additive effect is obtained on bacteria from the use of subinhibitory concentrations of streptomycin and penicillin.<sup>6</sup> Competitive excretions result in slightly higher drug levels when both agents are parenterally administered simultaneously. Streptomycin therapy alone or in combination with penicillin is warranted in the occasional instance of bacteremia due to gram positive cocci where a favorable response to maximal doses of penicillin is not obtained. Streptomycin is indicated also in the therapy of subacute bacterial endocarditis due to penicillin fast nonhemolytic streptococci and gram negative bacilli. Therapy must be continued beyond three weeks, and one must be prepared to accept the risk of labyrinthine disturbances from streptomycin toxicity developing in the occasional patient. On the basis of these experiences, the prerequisites for successful streptomycin therapy of bacteremia are that (1) the organism is sensitive to the drug, (2) acute endocarditis has not yet developed, (3) accessible foci of infection are adequately drained, and (4) the dosage of streptomycin is at least 2 Gm per day, the interval between doses is spread so as to provide bacteriostatic blood levels, and the duration of treatment is long enough.

*Infections Involving the Central Nervous System*—Streptomycin is now regarded as the drug of choice in the treatment of bacterial meningitis due to susceptible organisms.<sup>1</sup> The results in the U S Army series<sup>13</sup> in general, have been very favorable especially when treatment was started early in the disease. Of sixteen patients treated, twelve responded favorably and recovered while four died. Deaths were due to (1) use of streptomycin for *in vitro* resistant *Cryptococcus hominis* infection, (2) advanced sepsis, (3) loculation of pus in the base of the skull in two cases. It is important to remember in the management of the patient with meningitis that streptomycin, like penicillin, diffuses poorly into the cerebrospinal circulation following parenteral administration and that intrathecal injections must be combined with the intramuscular route of administration. The sulfonamides are the only effective bacteriostatic drugs which pass the blood meningeal barrier in effective concentrations. For this reason it may be desirable to combine oral sulfadiazine with streptomycin and/or penicillin therapy.

The scheme of treatment employed in the treatment of meningitis in adults is as follows. When the diagnosis of meningitis due to gram negative bacilli has been established, 50 to 100 mg (average dose, 1 mg per kilogram) of streptomycin dissolved in 10 cc of sterile physiologic saline are administered immediately by the intrathecal route. The injection is repeated every twenty-four hours until the patient has recovered and negative cultures have been

with the ulceroglandular type.<sup>8</sup> Pneumonia was a complication in three patients, in one of whom it was associated with nephritis. Streptomycin was administered to all patients intramuscularly at intervals of three or four hours in an average daily dose of 2 Gm. for periods of seven to fourteen days. Recovery was smooth in all patients, without relapses or complications. The most dramatic responses were obtained in patients with the typhoidal type of infection and with early ulceroglandular tularemia complicated by pneumonia. Infection in suppurating lymph nodes treated by aspiration and followed by one or two instillations of streptomycin subsided rapidly. Healing of open lesions in ulceroglandular tularemia was slow but progressive. The rate seemed related to the duration of the infection. The proposed local injection and topical applications of streptomycin to the ulcerated buboes to provide therapeutic concentrations of drug in relatively avascular tissues remain to be fully evaluated. These experiences support the conclusion of others that streptomycin is the most effective agent now available for the treatment of tularemia.<sup>1, 5</sup>

**Bacteremia**—Since 1943 the superiority of penicillin over all other available forms of treatment for bacteremias due to susceptible gram-positive organisms has been clearly demonstrated. Gram-negative bacilli, which are usually resistant to penicillin, also may on occasion invade the blood stream with serious consequences. The mortality rate from gram-negative bacteremias is not as high as in the case of gram-positive coccal bacteremias and the fatalities have been reduced further with the use of the sulfonamides. Streptomycin therapy promises to be even more efficient. The advantages lie in the prompt clinical responses and in the relatively trivial untoward reactions encountered with short courses of therapy.

Thirty-three patients with bacteremia were treated with streptomycin in U. S. Army Hospitals.<sup>10</sup> In the majority of these the infection arose in the urinary tract, other foci of infection in decreasing order of occurrence were sepsis in the peritoneal cavity, the female genital organs, and bone, including the middle ear and mastoid. Penicillin and/or sulfadiazine failed in twenty of the thirty-three patients before streptomycin therapy was employed. The dosage of streptomycin was between 2 and 4 Gm. a day in divided intramuscular doses, and treatment was continued for an average period of fourteen days. Thirty patients recovered and three died. The deaths in each instance were caused by advanced sepsis. Beneficial results were attributed to streptomycin in twenty-six of the thirty patients, and in the four remaining patients the results were of questionable value. In one of these the blood stream was cleared, but sepsis was unrelieved until surgical drainage of an abscess in the kidney was effected. In the second patient with bacteremia also originating in the kidney, blood cultures for *Klebsiella pneumoniae* were again positive after seven days of treatment. There was no response to a second course of the drug, and in all probability the bacteria had become resistant after the first course of therapy. There were no unusual features in the other two cases. One observation is common to the four failures: surgical treatment of the primary focus was not accomplished in conjunction with the chemotherapy.

Six patients with lobar pneumonia in which *A. pneumoniae* were the predominating organism in the sputa were treated with streptomycin. In each instance initial treatment was empirical, with penicillin alone or in combination with sulfadiazine. Each patient remained critically ill in the face of this therapy. After sputum cultures showed *A. pneumoniae* predominating, a change was made to streptomycin therapy. The dosage employed ranged from 0.2 to 0.4 Gm. given intramuscularly every four hours for an average of ten days. Response was striking in all instances, the temperature and pulse having returned to normal limits in seventy-two hours in three instances. There were no relapses. Complete clearing of the lungs, however, varied according to the duration of the infection. Certainly on the basis of this limited experience, streptomycin therapy is indicated in pneumonias caused by the *Klebsiella* organisms. Two cases of persistent *Hemophilus influenzae* infections in penicillin-treated pneumonias showed rapid resolution on streptomycin therapy. A patient with a hemolytic streptococcus pneumonia was developing progressive clinical signs in spite of seventeen days of normally adequate penicillin. The penicillin was discontinued and streptomycin, 0.25 Gm. intramuscularly every three hours, was given with beneficial results. Partial resolution was noted clinically and by x-ray in eight days, and complete resolution two days later. Another patient, who developed postoperatively a type IX pneumococcus pneumonia with bacteremia showed no response whatever to streptomycin. The organism was drug fast. Therapy with a combination of penicillin and sulfadiazine was successful. In two children, no dramatic alteration of the course of the disease attended streptomycin therapy in pertussis with bronchopneumonia. Streptomycin was of no value in the treatment of one patient with atypical pneumonia.

Gram negative bacilli are infrequent primary causes of empyema. They usually are present in mixed culture with the more commonly causal gram positive cocci. The treatment of pure coccal empyema by intrapleural injection of penicillin and by surgery has yielded very satisfactory results. Streptomycin has been tried in only a small series of patients, either alone or in combination with penicillin therapy. The results as a whole are not spectacular. This, in all probability, is due to the fact that streptomycin activity is markedly inhibited in exudates which are acid in reaction. It seems important to emphasize the fact that in all the cases in which improvement occurred the drug was employed in conjunction with adequate surgical drainage. A possible advantage of the combination of streptomycin with penicillin in empyema may be the more effective action of penicillin on gram positive organisms by virtue of the suppressive action of streptomycin on penicillinase-producing gram negative bacilli. In two instances of lung abscess and one instance of bronchiectasis a combination of penicillin and streptomycin, administered by nebulization and the intramuscular route, was considered beneficial. Three patients with bronchiectasis were treated only by nebulization and parenteral streptomycin without effect. Two patients with pleuritis, one of undetermined origin and the other secondary to peritoneal suppuration also were treated. There was no beneficial effect in the former patient. Improvement resulted in the latter after the peritonitis was controlled by combined streptomycin and penicillin therapy.

obtained. In addition, 0.5 Gm of streptomycin is administered intramuscularly every four hours. Treatment with sulfadiazine and penicillin may be carried on simultaneously. The total duration of treatment with streptomycin should not exceed seven days.

Streptomycin occupies a place similar to penicillin in the chemotherapy of extradural and solitary brain abscess when caused by susceptible gram-negative or polybacterial infections<sup>13</sup>. The technique of aspiration of pus through a trephine followed by injection of the drug (1 per cent saline solution) through a catheter produces satisfactory results except where bone is involved and sequestrectomy is required. Systemically administered drug should be given simultaneously with the local instillations to protect against dissemination and spread of the infection. It is emphasized that streptomycin does not reach the brain in assayable amounts and that therapeutic levels at the site of the infection can be achieved only by direct injection. Of eight patients with solitary brain abscess with varied bacteriology and primary causes receiving surgical drainage and/or wound revision together with combined systemic and local streptomycin therapy, recovery occurred in seven. The eighth patient died of sepsis.

*Suppurative Infections of the Ear and Mastoid*<sup>13</sup>—Five cases of otitis externa due to susceptible gram-negative bacilli and gram-positive cocci responded favorably with three or four applications daily of cotton packs soaked in 1 per cent sterile aqueous streptomycin solution. Chronic otitis media of mixed bacterial etiology also was treated with topically applied streptomycin, together with parenterally administered drug in a few instances. Of nineteen patients seventeen were benefited, while in two instances of *Ps. aeruginosa* infection no improvement resulted. Combined therapy seems rational because of the possibility of not contacting all reaches of the infection by either route alone. Five to seven days of treatment seem adequate for the majority of cases. Neither local nor systemic toxic phenomena have been observed from topical applications of streptomycin to the ear.

There may be a place for streptomycin alongside penicillin in the surgical and postoperative management of mixed infections of the mastoid. A patient with mastoiditis with lateral sinus thrombosis recovered without sequelae after eventration of the clot with parenteral streptomycin protection.

It is emphasized that streptomycin therapy can be a valuable addition to the therapeutic armamentarium for infections of the ear, meninges, and brain only when the organisms are susceptible to the drug and when sound proved surgical principles are strictly adhered to. There is no contraindication to the concurrent use of penicillin or sulfonamides and streptomycin.

*Infections of the Respiratory Tract*—Pneumopulmonary infections in which gram-negative bacilli are implicated have been treated with sulfonamides and type-specific antisera with inconstant success. These forms of therapy, therefore, leave much to be desired. Streptomycin has been evaluated in predominantly gram-negative and other types of lesions involving the respiratory tract<sup>1, 2, 5</sup>.



Two patients treated prophylactically after receiving stab wounds of the abdomen and chest did not become infected

One case each of blastomycosis, actinomycosis caused by *Nocardia asteroides*, and moniliasis was treated with streptomycin. Sustained improvement and remission lasting for an eight months' period of observation were observed in the patient with blastomycosis. The patient with actinomycosis showed remission only while under treatment. The organisms became drug-fast and the disease progressed when therapy was discontinued. No beneficial effect from streptomycin therapy was noted in the patient with moniliasis. Six patients with chronic bronchitis receiving streptomycin and/or penicillin alone or in combination both by the aerosol and intramuscular routes showed elimination of the gram-negative organisms and temporary clinical improvement which was sustained only in those receiving both penicillin and streptomycin. Asthma, Hodgkin's disease, pulmonary sarcoidosis, and other conditions of unknown etiology were not benefited by streptomycin. (Asthma, two patients, pulmonary sarcoidosis, seven, Hodgkin's disease, two, acute pericarditis and rheumatic heart disease, one each)

*Brucellosis* <sup>11</sup>—Twenty-nine patients with brucellosis have been treated with streptomycin, of whom sixteen had acute and thirteen had chronic cases. Blood cultures were positive for *Brucella* in fourteen of the sixteen patients with acute cases and a bacteremia was present in two with chronic cases. The organisms were very sensitive in vitro to streptomycin. The dosage of streptomycin varied between 1 to 2 and 6 Gm daily (two patients) for an average period of fourteen days. None of the patients with "chronic" brucellosis received any benefits whatsoever from streptomycin therapy. Of those with acute cases, only two of the twelve treated with streptomycin alone had fairly prompt remissions and negative subsequent blood cultures. The bacteremia disappeared in some but not in all of the subjects while the drug was being administered. Exacerbations occurred in five of the twelve patients.

Detailed study of one of the patients under treatment led to the support of the hypothesis that the foci of infection in brucellosis were not penetrated by streptomycin administered parenterally. Oral sulfadiazine, which earlier in the patient's disease was found to be ineffective, was given in addition to the parenteral streptomycin. A prompt response was obtained and the patient has remained well for eighteen months. Five additional patients with acute brucellosis (positive blood cultures for *Brucella* in four) also received combined streptomycin (3 Gm daily) and sulfadiazine (6 Gm daily) for an average period of fourteen days. Two patients are symptom-free at this writing. There were recurrences in two others six and twelve weeks, respectively, after therapy was discontinued. The fifth patient, a 24-year-old woman from whom no *Brucella* were cultured, was unaffected by the combined therapy.

#### *Infections of Intestinal Origin —*

*Typhoid Fever* <sup>11</sup> Six patients with typhoid fever were treated. The combined oral and parenteral route was used in three patients, and the parenteral route of administration alone in the other three. No remarkable results were achieved. In only one patient, a 5-year-old child who was given the usual adult

dosage (proportionately three times as great a dose as given the other patients), did the fever end abruptly enough to suggest a response to streptomycin therapy. In two patients who were asymptomatic typhoid carriers, combined oral and parenteral streptomycin therapy did not eliminate typhoid bacilli from the feces. A third carrier, a recent patient with typhoid fever with typhoid periostitis and positive bile cultures, was benefited by resolution of the bone lesion and elimination of *Escherichia typhi* from all cultures.

***Shigella and Salmonella Infections***<sup>11</sup> Beneficial effects were noted in ten patients with bacillary dysentery caused by *Shigella sonnei* in four and by *Shigella flexneri* subtypes in six, the majority having been treated previously with sulfonamides. Duration of therapy varied from one to twelve days. The most striking results were obtained in those patients treated during the first attack of illness and in those patients who received a combination of oral and intramuscular streptomycin. The symptomatic improvement was associated with disappearance of the dysentery bacilli from the stools. No relapses were recorded in this series of patients.

Two patients with acute gastroenteritis and blood cultures positive for *Salmonella* organisms were well at the end of seven days' treatment with streptomycin given parenterally and orally.

Oral streptomycin (100 mg per kilogram daily for four to seven days) treatment of three infants with *Salmonella* diarrhea resulted in remission of symptoms and in cultures negative for *Salmonella* for a three week follow up period. Of five patients with recurrent diarrhea showing *Salmonella* organisms, two received oral streptomycin without effect. One of these and three others of this group when treated with combined oral and intramuscular streptomycin obtained symptomatic relief and cultures remained negative for *Salmonella* species. In follow up these patients had mild abdominal cramps and loose stools, but *Salmonella* did not reappear.

***Colitis***<sup>11</sup> Of the patients with nonspecific ulcerative colitis, nine of those in the acute active phase with systemic manifestations had remissions of symptoms while receiving combined oral and parenteral streptomycin therapy, but the results were not permanent and there was no striking improvement as evidenced by sigmoidoscopic examination. The amelioration of symptoms obtained by streptomycin therapy may be valuable in restoring the patient sufficiently so that surgery can be more safely undertaken. Seven patients in the chronic static phase of colitis showed no improvement whatsoever. In our experience the use of streptomycin is not justified in the medical management of idiopathic ulcerative colitis.

Streptomycin therapy was tested in two patients with amoebic colitis and found to be of no value.

***Infantile Diarrhea***<sup>11</sup> Thirteen infants suffering from epidemic diarrhea of unknown etiology received oral streptomycin therapy in a dosage of 0.1 Gm per pound per day incorporated in the milk formula. At the start of treatment all were in shock and severely dehydrated. It seemed that streptomycin was the determining factor in saving at least four of the ten survivors. Vigorous fluid and protein replacement were important features of the therapeutic program.

*Peritonitis* Sixty-three patients have been treated with streptomycin for peritonitis of fecal origin<sup>14</sup> Fifty-eight patients recovered and five died Three of the fatalities were instances of generalized fibrinopurulent peritonitis, and streptomycin was added to the therapy after the patients had become moribund Eighteen of the patients who recovered were treated with streptomycin alone, thirty were treated with streptomycin and penicillin, and ten were treated with streptomycin, penicillin, and sulfadiazine The most striking response was associated with the use of streptomycin in early spreading peritonitis, whether used alone or in combination with the other antibiotic agents Approximately the same course was seen in the resolution of established peritoneal suppuration under streptomycin therapy as under massive doses of penicillin The infections that had already localized showed less consistent response to streptomycin The impression was received that more rapid resolution of these lesions resulted with combined streptomycin and penicillin therapy The most consistently beneficial effects were pursuant to the concurrent intramuscular administration of 0.3 Gm of streptomycin and 100,000 units of penicillin every three hours It seems likely that the complementary antibacterial spectra of these two antibiotics will favor their combined use for peritonitis of polybacterial etiology

*Wound Infections*—This unit is especially concerned with identifying the indications for, the dosage of, and the adjuvant utility of streptomycin in the treatment of impending and established wound infections At this writing, data on the results of therapy in sixty-one patients with infections of soft tissues have been examined critically Although these data are insufficient to establish precise clinical standards, these cases are sufficiently representative to indicate certain possibilities and limitations of the antibiotic Streptomycin apparently has its chief field of usefulness in the therapy of cellulitis Particularly favorable results were obtained in acute mixed gram-positive and gram-negative infections which were not responding to, or which developed in the face of, penicillin therapy Another use for the drug appears to be the gram-positive coccal infection which has not responded to maximal doses of penicillin within seventy-two hours, or earlier if in vitro evidence of penicillin fastness has been secured A third indication for use is the occasional case of streptomycin-sensitive gram-positive coccal infection developing in the patient with idiosyncrasy to penicillin It is worthy of note that the mixed infections in this series occurred predominantly below the level of the diaphragm No strikingly beneficial results have been noted following topical application of streptomycin to wounds It is well to recall that the mere presence of gram-negative organisms in a wound is not per se an indication for streptomycin therapy These bacteria are a feature of necrotic tissue and are eliminated only when all such tissue is removed The only indication for topical streptomycin therapy is in conjunction with débridement The dosage of streptomycin when used alone has been 2 to 3 Gm per day When used in combination with penicillin, the dosage recommended at present is 0.25 Gm together with 50,000 units of penicillin, given intramuscularly every four hours Investigations to establish the optimum dosage of the combination are now being conducted

Two hundred and fifty eight complex wound infections of battle casualties were also evaluated. Almost all of them were associated with chronic osteitis, fibrosis, and diminished local circulation. Usually a mixed bacterial flora was present. *Staphylococci* and *streptococci* were almost constantly identified and *clostridia* were present in approximately one fourth of the patients. A variety of gram negative bacteria including *proteus coli*, *pseudomonas* and *aerogenes* were associated. Of these patients ninety eight were considered to be benefited and one hundred and sixty were considered as not benefited by streptomycin.

The main task of arresting chronic infection in bone is surgical, not chemotherapeutic. Penicillin and to a lesser degree streptomycin, relieve the surgeon's anxiety about sepsis and protect against spread of infection attending traumatic surgical procedures. Chemotherapy has a definite place in the post operative management of the bone cavity. Bacteria harbored in residual dead tissue and blood clots perpetuate infection unless eradicated. Only local chemotherapy can soak these tissues in high enough concentration. In our experience, combined penicillin and streptomycin therapy is distinctly advantageous in the management of chronic osteitis from the time of sequestrectomy through the time of diverse reconstructive procedures.

*Tuberculosis*—Streptomycin has now been demonstrated to be a valuable agent in certain forms of tuberculosis. In the pulmonary forms, administration parenterally of streptomycin in daily doses of 20 to 30 Gm. a day for periods up to one hundred and twenty days results in improvement above and beyond that expected from bed rest alone in 50 per cent of the patients. The exudative phase of the infection responds most readily, while fibrotic lesions remain unchanged. Streptomycin in conjunction with collapse therapy enhances the prospects of clinical arrest of the disease in properly selected cases. This antibiotic affords protection against spread of infection postoperatively following thoracoplasty. The results in pharyngitis, laryngitis, and bronchogenic tuberculosis are generally encouraging. Streptomycin has some effect in military tuberculosis and in tuberculous meningitis, though relapse following withdrawal of the drug must be anticipated. Streptomycin in combination with appropriate surgical treatment seems indicated in hematogenous tuberculosis with soft tissue involvement and in bone tuberculosis though observations are still limited.

Streptomycin therapy of tuberculosis is not without danger. There is some relationship between toxicity and dosage and duration of therapy (see Untoward Reactions). Central nervous system effects, especially on the vestibular portion of the eighth cranial nerve, are a common feature of the courses of therapy necessary to affect tuberculous lesions. These effects are slow to reverse themselves and some damage may be permanent.

#### UNTOWARD REACTIONS

The case records of eleven hundred and fifty three patients in this series were reviewed and the untoward reactions were tabulated. The over all incidence of side effects was 27.9 per cent (three hundred and twenty two patients). In discussing these reactions from the standpoint of incidence, severity, and cause,

it is emphasized that this series was begun in 1945, when streptomycin was relatively impure. As noted by McDermott,<sup>4</sup> certain untoward reactions are probably the effects of impurities in the streptomycin, while others are caused by the streptomycin molecule itself. It might be pointed out that a number of the side reactions recorded here are now only of historic interest.

*Neurologic Disturbances*—These are the most important. There are two types, the persistent or slowly regressing, and the transient. Of the transient reactions, circumoral pallor and tingling of the face and extremities were recorded fifty times. This reaction has appeared as early as following the first intramuscular injection. Disappearance in the face of continued therapy is usual. Flushing of the skin is an occasional concomitant. Tinnitus, which also may appear early, was noted in twenty-eight patients. Vertigo may be either transient or persistent and its appearance should be reason for caution. Neurologic examination is indicated when this symptom appears. Transient vertigo appeared usually between the third and tenth days of treatment and lasted one or more days. It is recorded thirty times, which may be an underestimation of the incidence. Persisting vertigo, which was accompanied by an ataxic gait and absence of vestibular response to caloric tests, was noted in fifty-six patients (5 per cent). All were on courses of therapy ranging from twenty-one to one hundred and twenty days. The incidence was highest in those treated for one hundred twenty days. The earliest appearance of this disturbance recorded is in a patient receiving 3 Gm of streptomycin daily for fourteen days. The incidence in eighty-one patients receiving 0.5 Gm of streptomycin every four hours intramuscularly up to a total of 50 Gm was 3.7 per cent.<sup>3</sup> This reaction is slow to reverse itself. Partial deafness was recorded in twelve patients (1 per cent), all were patients with tuberculosis on a one hundred twenty-day program.

*Sensitizations*—Dermatoses occurred in thirty-five patients. These reactions were accompanied by pruritis and usually by fever and eosinophilia. The manifestations varied from localized macular eruptions over the flexion creases of the forearms or over the sites of injection to generalized rashes. In six patients urticaria presented. The usual appearance of sensitization reactions was between the fifth and tenth days of therapy. Withdrawal of the drug always resulted in subsidence. Reappearance did not always follow a second course of treatment. In some patients the reaction gradually disappeared in the face of continued streptomycin administration in conjunction with antihistamine drug therapy. There were four instances of exfoliative dermatitis, all in patients with tuberculosis. This reaction is serious and requires prompt withdrawal of the drug. Disappearance is gradual.

*Urinary Phenomena*—Albumin and microscopic examinations were made on a series of forty patients receiving parenteral streptomycin therapy. Slight albuminuria occurred in all instances and cylindruria in twenty-eight. These findings were not present at the conclusion of treatment. Renal irritation in no instance was regarded as sufficiently important to interrupt therapy. Tests for azotemia should be carried out on patients with evidence of prior renal

damage or on patients whose course of therapy is extended beyond two weeks.

*Histamine like Reactions*—These are transient and relatively uncommon with presently used streptomycin. In this series, headache was recorded fifty five times, nausea and vomiting, twenty three times, arthralgia, thirty four times, and asymptomatic fall of 20 mm Hg or more of systolic blood pressure, ten times.

*Local Irritations*—This complaint, formerly universal, is now less frequently encountered. In our experience, the intramuscular injections of present batches of streptomycin are still followed by some pain and soreness, more so than in the use of penicillin, but we no longer make a practice of diluting the streptomycin in procaine solution. It is our routine to rotate the sites of injection among the deltoids, the hips, and the thighs.

On the basis of this experience, it is concluded that the use of highly purified streptomycin is justified in the treatment of all types of serious infections due to susceptible bacteria. Treatment certainly can be continued with safety for ten days.

#### SUMMARY

1 Experiences with streptomycin therapy in twelve hundred patients with infections treated in U. S. Army Hospitals have been presented.

2 The wide variation in sensitivity of bacterial species to streptomycin emphasizes the importance of checking the causative organisms in the laboratory for their susceptibility prior to therapy wherever possible. The cardinal principles which govern in the various fields of medicine and surgery must be fulfilled in conjunction with the chemotherapeutic program. Failure of the host to kill small numbers of resistant organisms or the presence initially of many resistant forms, will result in ineffective drug therapy in many instances and in the persistence of drug fast bacteria. Drug fastness to streptomycin is specific and is not carried over to other chemotherapeutic agents.

3 Present indications for the drug include gram negative urinary tract infections, tularemia, bacteremia, pneumonia, and meningitis due to susceptible organisms, and certain otolaryngologic conditions associated with gram negative bacteria.

4 Bacterial pneumonitides caused by streptomycin susceptible organisms are likely to show good responses to streptomycin therapy. Results in bronchiectasis and empyema have not been spectacular.

5 In limited experience, acute brucellosis with bacteremia was favorably influenced when streptomycin was given in combination with sulfadiazine. The drug is of no value in "chronic" brucellosis.

6 Results in typhoid fever have not been encouraging.

7 Streptomycin shows promise in the treatment of bacillary dysentery refractory to sulfonamides and in some types of *Salmonella* infection. It may be influential in bringing about a remission in active idiopathic ulcerative colitis with severe secondary infection caused by susceptible organisms. The drug is not useful against amoebic colitis.

8 Striking results have been obtained with streptomycin therapy in a few patients with early diffuse peritonitis, but the most consistent beneficial

effects were noted when the streptomycin was given in combination with penicillin

9 Streptomycin is complementary to penicillin in the surgical management of wound infections, and may be of value in certain lesions heavily infected with fecal organisms

10 A general statement has been made relative to the preliminary results of streptomycin therapy in tuberculosis

11 The incidence and type of untoward reactions encountered in this series have been presented. It is concluded that the toxicity of presently available streptomycin is sufficiently low to justify its use in serious infections against which it has been shown to be effective

#### ADDENDUM

Seven patients in all have completed a course of therapy to date, that is, 3 Gm of streptomycin per day together with 6 Gm of sulfadiazine, for an average of twenty one days. The results of therapy are as follows. Two patients are well and symptom free eighteen months following treatment. A third patient has been free of complaints for nine months except for arthralgia of both knee joints and the proximal phalanges of the third and fourth fingers of the right hand. In this patient the agglutination test for *Brucella* organisms was positive once in a dilution of 1:160, but negative when repeated. Opsonic-trophic test was performed by the National Institute of Health and was reported positive in 1:40 strength. This patient has been seen by a number of consultants for joint diseases who feel that the arthritic process is rheumatoid rather than due to brucellosis. In two other patients relapses occurred on the seventh and twelfth weeks after treatment. A second course was not given because both patients suffered moderately severe vestibular disturbances. A sixth patient is well at this writing, but the period of observation has been for a duration of only three months. A seventh patient, in whom positive blood cultures were never obtained, was uninfluenced by the combined therapy.

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# SEROLOGIC STUDIES OF INFLUENZA MADE IN BOSTON DURING THE WINTER OF 1945-1946

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THE 1945 epidemic of influenza B which was recognized in many parts of this country and in other countries<sup>1-13</sup> reached its peak of incidence in and around Boston during the latter half of December, 1945. This epidemic offered an opportunity to make some serologic observations during and after an outbreak of influenza B similar to those that were made in this laboratory during and after the outbreak of influenza A that occurred here in December 1943.<sup>14</sup> The reactions of the viruses obtained during the height of the 1943 epidemic and the antibody response to characteristic infections occurring at that time indicated that the epidemic cases were caused chiefly by strains of influenza A virus similar to PR8. The postepidemic cases of clinical influenza on the other hand, were atypical not only with respect to the viruses which were isolated from some of them but also with respect to their antibody responses to both their own strains and to the standard PR8 strain. There was no evidence of infection with influenza B in any of the cases studied.

After the influenza A epidemic it was also found that the sera of most persons giving a characteristic history of influenza during the period of the epidemic had significantly elevated titers of antibodies to PR8 demonstrable for as long as twelve weeks after the onset of these symptoms. Such elevated titers could not be found during the same period in the sera of persons who denied having had any symptoms of acute respiratory infections or who had had symptoms which could be distinguished readily as those of the common cold and not influenza.

Of particular interest were the high titers of antibodies to PR8 that were demonstrated in severe cases of bacterial pneumonia which occurred during and shortly after the epidemic and in which there was an antecedent history of clinical influenza. Those findings and the isolation of influenza virus from the lungs of three patients with fatal cases suggested that the occurrence and severity of the pneumonia in such cases were related to the influenza virus infection.

The present paper deals chiefly with the results of serologic tests for influenza antibodies in cases of clinical influenza and other acute respiratory infections which occurred during and for a few weeks after the epidemic prevalence of influenza B. The results of tests made on two other groups of sera are also included: one group consisted of paired sera obtained during and shortly after

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the height of the epidemic from persons who were entirely free of illness, and the other consisted of single samples from a somewhat larger group of persons obtained a few weeks after the epidemic had subsided. The results of attempts at isolation and identification of virus from patients with acute cases in this epidemic and the serologic findings in cases of pneumonia are left for separate consideration elsewhere<sup>1-16</sup>

#### MATERIALS AND METHODS

*Serologic Tests*—Both inhibition of chicken cell agglutination and complement fixation tests were carried out in this study. The antigens employed included the PR8 strain\* of influenza A and the Lee strain\* of influenza B and two strains, WC and MF, of influenza B isolated from patients early in the course of this study. The details of the collection and preservation of the sera, the serologic methods, and the manner of reading and recording the end points were identical with those used in a collateral study<sup>12</sup>

*Cases Studied*—Included in the present studies were members of the hospital and laboratory staff, both with and without illness, and patients admitted to the regular adult medical wards of the hospital between the middle of December, 1945, and early in March, 1946. Control sera obtained early in the study were available from some of the hospital and laboratory personnel who subsequently developed respiratory infections. With a few exceptions (noted in Table III) patients admitted to the hospital with clinical and x-ray findings of pneumonia are omitted here, since they are considered separately elsewhere<sup>16</sup>

*Clinical Findings*—The clinical features of the cases of influenza were quite characteristic and were similar to those observed in the Needham outbreak<sup>12</sup>. In the patients who were seen during the acute illness, an attempt was made to evaluate and classify the clinical findings at that time. This was done again in all of the patients when all of the clinical findings were available and entirely without reference to the serologic data. Those with findings that fit the characteristic picture of the epidemic disease, namely chills or chilly sensations, fever, prostration, headache, eyeball soreness, generalized aches, mild sore throat, and some cough, were classified as having clinical influenza and the severity of their illness was graded 1 plus, 2 plus, or 3 plus. Patients with a minimum of fever and systemic symptoms but with moderate or marked coryza, with or without cough, were classified for convenience as having "colds". A third type of illness was also recognized in which the systemic symptoms resembled somewhat those of influenza but the patient suffered chiefly from anorexia, nausea, vomiting, and diarrhea and had no symptoms referable to the respiratory tract—so-called "intestinal flu".

#### RESULTS

The cases studied have been arranged for convenience into two main groups, one including patients with acute respiratory infections and the other those without such infections. The former was further arranged into four subgroups to include patients in whom there was serologic evidence of infection with

\*Originally obtained from Dr. Thomas Francis, Jr., Ann Arbor, Mich.

influenza B, patients in whom studies of acute and convalescent phase sera failed to yield evidence of infection with either influenza A or B patients with severe acute respiratory infection, including five who had fatal cases from whom sera were available only during the acute phase of the illness and finally those in whom there was serologic evidence of infection with influenza A. The group without respiratory illness was composed of two subgroups a small number of hospital and laboratory workers from whom control sera were obtained in the second week of December and again about three weeks later and in whom no respiratory illness developed during the course of this study and hospital personnel and ward patients who were under treatment at the time for nonfebrile illness other than acute respiratory infections and who each contributed a single specimen of serum toward the end of this study. The results of the serologic findings in each of these groups of cases will be considered separately. They will then be summarized in order to bring the findings in the various groups into proper perspective.

#### PATIENTS WITH ACUTE RESPIRATORY INFECTIONS —

*Group I Patients With Serologic Evidence of Influenza B Infection* — The relevant findings in twenty three such patients are listed in Table I. In each of these patients there was at least a fourfold rise in titer of the influenza B antibodies and much greater rises were noted in almost every instance. For the most part rises of similar grade were demonstrated by both the agglutinin inhibition and the complement fixation tests with the Lee strain and with the two epidemic strains. There were very few discrepancies, as for example in Patients 4, 7 and 9 in whom the agglutinin inhibition test with one or another of the B viruses showed little or no rise while significant rises were noted by complement fixation with the same virus or, indeed by the same test with another of the B strains.

There was no appreciable rise in titer of PR8 antibodies demonstrable by either of the tests used in any of these cases. The initial titers of these antibodies were low except for a few elevated titers of agglutinin inhibition.

Each of these patients with the exception of the first one, had an infection which was considered to be characteristic of the epidemic influenza and which began between Dec 12 and Jan 3. The illness however varied considerably in these cases both with respect to the height and duration of the fever and the severity of the systemic symptoms. Leucocyte counts were done during the acute illness in most of these cases. Only two patients showed elevated counts 11,700 in one and 14,000 in the other and both of these patients had moderately severe tracheobronchitis without physical or x-ray signs of pulmonary infiltration. The leucocyte counts in slightly more than half of the remaining cases ranged between 4,200 and 6,500 and in the others they ranged between 7,000 and 10,000.

The only patient without the characteristic findings of clinical influenza (Patient 1) had a mild cough which began Dec 6 lasted for ten days and was not accompanied by fever or systemic symptoms. In this case a rise in the B antibodies occurred from low titers after the eighth day. In five additional

TABLE I SEROLOGIC FINDINGS IN PATIENTS WITH EVIDENCE OF INFLUENZA B INFECTION

PATIENT	CLINICAL INFLUENZA		AVERAGE TITER OF INFLUENZA ANTIBODIES								
	DATE OF ONSET	SEVERITY	DATE OF SERUM	PF8		LEE		WC		MF	
				AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
1	12/ 6	C	12/14	13	6	4	4	6	10	8	10
2	12/12	+++	1/ 2	13	6	10	25	12	40	32	40
			12/15	8	6	5	3	2	2		
3	12/13	++	12/27	6	6	12	56	46	64		
			12/14	24	2	15	3	3†			
4	12/13	+	1/ 2	24	12	56	44	24			
			12/14	15	7	12	6	16	7		
5	12/14	+	1/ 2	15	9	32	16	16	32		
			12/14	24	2	11	4	2	3	5†	6
6	12/15	+	12/27	27	3	115	272	40	180	64	192
			12/26	16	10	192	56				
7	12/16	++	1/ 9	16	7	512	384				
			12/23*	32	9	5	2	2	2		
8	12/17	+++	1/20	32	12	10	52	12	64		
			12/18	18	12	10	52	16	7	8	6
9	12/18	+	1/ 5	32	8	278	145	256	128	128	96
			12/18	2	5	2	2	2	2		
10	12/18	+++	1/ 4	2	3	4	28	8	28		
			12/21	20	32	2	2	2	2	3	2
11	12/ 8	C	12/28	20	32	1024	192	48	256	96	192
			12/23	256	32	6	5	2	7	3	7
12	12/20	++	12/26	128	28	64	192	160	320	64	256
			11/20	20	8	21	6	8	5	5	5
13	12/21	++	12/21	20	7	117	28	64	24	32	16
			12/21	64	10	14	4	4	3	3	3
14	12/24	++	1/16	64	9	59	13	32	12	12	10
			12/29	8	12	5	2	2	2	2	2
15	12/17	D	1/ 2	6	8	21	28	16	32	12	12
			12/26	32	24	4	2	2	2	2	2
16	12/25	++	1/ 4	24	32	192	104	64	96	64	56
			12/26	16	24	4	2	2	2	4	2
17	12/27	++	1/ 6	16	40	36	480	256	896	256	896
			1/ 2	3	3	3	5	2	8	2	6
18	12/27	+++	1/8	10	8	132	112	48	128	96	128
			1/10	8	3	6	24				
19	12/27	C	1/10	8	2	24	192				
			1/ 2	27	9	5	9	4	12		
20	12/30	++	12/30	51	16	64	44	32	56		
			12/31	6	3	6	2	3	2	4	2
21	12/ 4	C	1/ 8	3	5	64	165	64	224	64	128
			1/ 3	64	24	8	4				
22	1/ 1	+++	1/10	48	20	320	512				
			1/ 5	8	4	32	14				
23	1/ 3	++	1/11	8	3	2048	896				
			1/ 4	128	19	32	7				
			1/22	96	26	192	96				

Only two sera are listed in each case. Intermediate and later sera were obtained and studied in almost every case but these are omitted from this table and from Tables II and III. Those selected for inclusion in these tables showed the maximum changes observed.

Symptoms considered typical of influenza are graded according to severity as + ++ or +++ C coryza and/or cough without systemic symptoms D gastrointestinal symptoms pre dominant.

\*Relapse with increase in severity

†Homologous virus

patients there was another distinct illness preceding the one considered to be influenza. This antecedent illness was classified as a common cold in four instances and as "intestinal flu" in the fifth. The interval between the onset of the two illnesses was three days in one patient and from eight to thirty one days in the others, and the initial serum was obtained up to three days after the onset of the second illness. In none of these cases, however, was a significantly elevated titer of antibodies for either the A or B viruses demonstrated in the acute phase serum to suggest a possible earlier influenzal infection. Patient 11 may be an exception since an elevated titer of agglutinin inhibition was demonstrated in the first blood with the PR8 strain. The corresponding complement fixation titer, however, was not correspondingly elevated in that serum and the interpretation of this finding must remain in doubt. Patient 6 was admitted on the eleventh day after the onset of what was considered to be a typical but mild influenza from which he seemed to improve until a relapse occurred with similar but somewhat more severe symptoms three days prior to entry. The Lee antibodies were elevated in the initial blood obtained on entry but later showed a significant rise suggesting that the initial infection was indeed influenza B.

*Group II Patients Without Evidence of Influenza A or B Infection*—For reasons which will become apparent later, the patients in whom tests of acute and convalescent sera failed to disclose any evidence of infection with either influenza A or B were divided into two groups according to the date of onset of their illness. Those in whom the illness began before the second week in January corresponding to the dates of onset in the cases just described, will be considered first. The few patients whose illness began after the middle of January will then be discussed.

*A Patients With Onset of Illness Prior to Mid-January Whose Sera Failed to Show an Antibody Rise* There were twenty one such patients and the findings are shown in the first part of Table II (Patients 24 to 44). There was no antibody rise whatever in any of these cases to PR8, Lee B or either of the epidemic strains demonstrable by either of the tests used. The initial titers in these cases were low, for the most part and corresponded to those observed in the patients in whom rises in influenza B antibodies were demonstrated.

The illness in thirteen of these twenty one patients was similar in most respects to that observed in the previous group and was classified as influenza of varying severity. In the other eight patients the illness clinically either resembled the common cold (four cases) or the symptoms were predominantly gastrointestinal (two cases) or both of these types of illness occurred in succession (two cases).

*B Patients Whose Illness Began After Mid-January and Whose Sera Failed to Show an Antibody Rise* There were only eight patients in this group and the findings are shown in the lower part of Table II (Patients 45 to 52). In this small group there seemed to be a higher proportion of sera in which agglutinin inhibiting antibodies for PR8 were in the high range. The titers otherwise were similar to those observed prior to Jan 7. Each of the patients

TABLE II PATIENTS WITH ACUTE RESPIRATORY INFECTIONS LACKING SEROLOGIC EVIDENCE OF INFECTION WITH INFLUENZA A OR B

PATIENT	CLINICAL INFLUENZA		AVERAGE TITR OF INFLUENZA ANTIBODIES								
	DATE OF ONSET	SEVERITY	DATE OF SERUM	PR8		LEE		WC		MF	
				AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
24	11/25	C	12/14	80	18	44	1				
			12/11	80	48	60	13				
25	12/ 2	C	12/14	8	2	4	2				
			12/11	8	5	6	2				
26	12/ 7	C	12/20	32	20	24	2				
			12/24	32	24	32	2				
27	11/26	D	12/14	6	20	24	12				
	12/ 9	C	1/ 2	6	10	8	10				
28	12/11	++	12/17	13	13	19	6			6	6
			1/ 3	10	6	15	5			8	6
29	12/11	++	12/14	16		10	9	2	12	2	12
			1/ 5	16	4	10	10	2	14	2	12
30	12/11	++	12/16	4	7	4	7	2	4	3	5
			12/31	4	3	3	3	2	4	4	6
31	12/11	D	12/18	28	5	2	3				
			1/ 4	28	5	3	2				
32	12/12	++	12/12	96	80	72	16	3	10	3	12
			1/ 2	96	96	88	20	4	8	3	12
33	12/13	D	12/14	88	10	24	15	2	10	5	6
	12/14	C	12/28	48	20	34	27	5	24	5	9
34	12/14	C	12/17	2	2	2	2				
			1/ 2	2	2	2	2				
35	12/14	+	12/14	16	28	12	36	16	40	16	28
			1/ 2	16	28	14	32	16	40	16	32
36	12/18	D	12/18	16	6	5	3	2	3	6	3
			1/ 4	16	6	4	3	2	3	12	3
37	12/19	+	12/13	12	5	7	12	4	20	4	16
			1/ 2	12	10	9	8	3	12	4	14
38	12/19	++	12/18	16	23	2	32	24	24	6	20
			1/ 2	16	36		40	24	28	6	24
39	12/30	+++	12/28	16	24	6	6	2	6	6	8
			1/ 7	32	24		5	2	5	6	10
40	12/22	+	12/24	6	24		4	4	2	2	3
	1/ 4		1/11	6	16	3	4	3	2	2	5
41	12/23	+	12/14	8	5	14	6	4	10	24	10
			1/ 4	6	7	14	7	4	10	16	10
42	12/23	++	1/12	64	2	24	2				
			1/18	64	2	32	2				
43	1/ 3	++	1/ 3	80	28	72	24				
			1/15	80	28	72	24				
44	1/ 6	+	1/ 2	20	16	64	32				
			1/17	20	16	96	32				
45	1/18	+	2/ 4	64		12	2				
			2/14	128	6	12	2				
46	1/21	++	2/ 6	64	16	24	1				
			2/21	96	11	8					
47	2/18	++	2/20	112	20	28	4				
			3/ 5	171	32	27	4				
48	1/17	++	2/21	64	6	64	14				
	2/20	C	3/12	64	6	64	20				
49	3/ 4	+++	2/26	25	7	36	8				
			3/11	24	7	36	7				
50	3/ 6	+++	3/ 7	16	20	3	14				
			4/ 1	24	20	3	12				
51	3/12	++	3/1	192	28	32	8				
			4/ 2	192	28	24	7				
52	3/12	++	3/1	64	28	32	20				
			3/28	64	22	22	16				

Symptoms considered typical of influenza are graded according to severity as + ++ or +++ C coryza and/or cough without systemic symptoms D gastrointestinal symptoms pre dominant

had an illness which was characteristic of the epidemic influenza. One had a second illness classified as a cold which began one day prior to the time the initial blood was taken.

*Group III Findings in Acute Phase Sera*—There was a group of twenty patients with severe acute respiratory infections from whom sera were obtained during the acute phase of the illness, but later specimens were not available. Almost all of them had symptoms that began before mid January and were classified as those of influenza. In some of them these symptoms were followed by the typical findings of pneumococcal pneumonia. There were five deaths in this group but autopsies were not obtained in any of them. Agglutinin inhibition titers greater than 64 against PR8 were noted in two patients and against Lee in one patient, but the corresponding complement fixation titers were low in each instance. The remaining titers of antibodies to the PR8 and Lee strains in this group of cases were all low and corresponded to those found in the acute phase sera in those patients who later showed a rise against the B strains.

*Group IV Patients With Serologic Evidence of Influenza A Infection*—There were thirteen patients in whom the serologic findings indicated infection with influenza A. The relevant data in these cases are summarized in Table III. The onset of illness in all of these cases occurred between Jan 18 and Feb 3, except in Patient 53 whose symptoms began Dec 28. In five of these patients (Patients 56, 57, 62, 63, and 64) the initial serum obtained between the seventh and twenty-first day after the onset already had significantly elevated PR8 antibody titers. However, in contrast to the isolated instances of elevated titers of agglutinin inhibition by PR8 noted in the previous groups of cases in which there was no corresponding elevation in the titer of complement fixing antibodies obtained with the same virus, the elevated titers in the present five patients were demonstrated by both tests. In the remaining eight patients, significant and usually marked rises in antibodies occurred to the PR8 but not to the Lee virus.

Several attempts to isolate a virus from pharyngeal washings obtained during the first day of illness in two of the patients (Patients 54 and 60) were entirely unsuccessful.

There were two patients in this group in whom there was definite serologic evidence of successive infections, first with influenza B and then with influenza A. One of these, Patient 58, was originally admitted to the hospital for influenza which began Nov 30 and was accompanied by characteristic symptoms and physical and x-ray findings of atypical pneumonia. The patient recovered fully and later was readmitted because of a second attack of influenza, the symptoms of which began Jan 27. The titer of antibodies for the Lee strain had risen from 20 to 640 by agglutinin inhibition and from 3 to 1,792 by complement fixation during the first illness. These titers had already dropped appreciably at the time of the second hospital admission. There had been no rise in PR8 antibodies during the first attack.

The early symptoms in the original attack of influenza in the second patient, Patient 59, were quite characteristic of the epidemic disease and began on Dec 24. These were followed by a typical course of type 5 pneumococcal pneumonia which began abruptly on Dec 29. During this illness there was a marked rise

TABLE III PATIENTS WITH EVIDENCE OF INFLUENZA A INFECTION

PATIENT	CLINICAL INFLUENZA		AVERAGE TITER OF INFLUENZA ANTIBODIES				
	DATE OF ONSET	SEVERITY	DATE OF SERUM	PRS		LEE	
				AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
53	12/28	C	12/31	32	4	12	6
			1/11	256	96	12	5
54	1/18	+++	1/19	12	20	64	44
			1/29	36	60	56	44
55	1/21	++	1/4	28	5	3	2
			1/28	96	14	6	2
			2/26	160	28	8	2
56	1/24	++	2/4	256	160	60	16
			3/1	256	125	60	9
57	1/25	+	2/15	1024	112	32	4
58	1/27	++†	1/28	64	22	149	168
			2/14	256	112	144	104
59	?	0‡	1/16	32	2	1280	160
			2/27	192	64	32	20
60	1/30	++	1/31	12	2	6	10
			2/15	160	48	8	11
61	2/1	++	2/7	64	24	3	14
			2/14	2048	768	5	10
62	2/1	++	2/8	96	144	48	2
63	2/1	+++§	2/8	384	72	12	4
64	2/2	+	2/18	192	100	32	2
65	2/3	++	11/31	80	48	60	13
			2/26	352	104	32	11

Symptoms considered typical of influenza are graded according to severity as + ++ or +++ C coryza and/or cough without systemic symptoms O no symptoms

\*Bronchopneumonia onset 2/12 W B C 11900 etiology ?

†Had influenza with atypical pneumonia (onset 11/30) with rise in influenza B antibodies from 20 to 640 by agglutinin inhibition test and from 3 to 1792 by complement fixation test. Cold agglutinins could not be demonstrated in any of the sera

‡Influenza (onset 12/24) and type 5 pneumococcal bronchopneumonia (onset 12/29) with marked rise in influenza B antibodies (from titer of 4 in acute phase) No definite symptoms associated with influenza A infection

§Type 5 pneumococcal bronchopneumonia onset 2/6 W B C 6600

||Type 4 pneumococcal lobar pneumonia onset 2/2, W B C 3000

in antibodies to PRS but not to the Lee strain although the initial titers to both of these viruses were low. The patient was discharged from the hospital and subsequent specimens of serum were obtained when the patient returned on two occasions for checkup examinations. The sharp rise in PRS antibodies that occurred following this patient's discharge was not associated with any subjective or objective evidence of infection.

In all but one of the remaining patients the infections which occasioned the rise in influenza A antibodies could not be distinguished clinically from those which were associated with the rises in influenza B antibodies. In Patient 53 the illness was characterized by coryza, slight sore throat, and slight productive cough without fever or systemic symptoms, the leucocyte count was 8,700

#### PATIENTS WITHOUT ACUTE RESPIRATORY INFECTIONS —

*Group V Serologic Findings During the Influenza B Epidemic in Persons Without Respiratory Infections*—There was a group of eleven hospital and

TABLE IV RESULTS OF TESTS FOR INFLUENZA ANTIBODIES IN SERUMS OBTAINED BETWEEN FEBRUARY 16 AND 27, 1946, FROM HOSPITAL PATIENTS AND NORMAL PERSONS WITHOUT ACUTE RESPIRATORY INFECTION

PATIENT	RECENT HISTORY OF CLINICAL INFLUENZA*	AVERAGE TITER OF INFLUENZA ANTIBODIES								
		DATE OF SERUM	PR8		JEE		WC		MP	
			AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
66	0	2/16	32	10	16	8	6	20	4	20
67	C2	2/16	64	2	6	6	2	12	6	7
68	C1	2/16	256	12	4	2	6	20	6	20
69	C1 2	2/16	64	6	64	20	32	40	32	20
70	C1	2/16	96	12	128	32	48	48	64	40
71	0	2/17	128	12	64	16	4	12	4	6
72	0	2/17	96	6	8	3	24	7	2	6
73	C2	2/18	96	40	64	16	192	40	8	28
74	0	2/18	256	32	12	7	8	12	8	12
75	C1	2/18	48	4	16	8	8	14	4	12
76	C1	2/18	32	5	48	7	8	20	8	20
77	0	2/18	64	8	48	12	16	20	16	28
78	0	2/18	192	24	32	6	4	3	4	5
79	C1	2/18	48	16	24	20	8	28	6	32
80	C1	2/20	64	28	96	40	2	48	64	48
81	V	2/20	512	40	512	80	128	112	192	128
82	C1	2/20	12	3	32	2	8	7	16	10
83	C2	2/20	32	20	256	24	8	24	8	112
84	C1	2/20	128	2	64	16	8	40	16	32
85	C1	2/20	96	24	64	16	32	24	32	20
86	0	2/20	32	3	24	3	2	7	2	10
87	C1	2/20	64	16	128	12	16	29	16	20
88	C1	2/20	128	14	96	12				
89	C1	2/20	28	10	128	48				
90	C1 2	2/21	80	10	128	22				
91	C1 2	2/21	96	20	96	16				
92	C1 2	2/21	48	12	40	16				
93	0	2/21	8	20	8	20				
94	C2	2/21	80	20	7	20				
95	C1 2	2/21	80	14	8	2				
96	0	2/21	80	24	12	10				
97	C1	2/21	96	20	4	2				
98	F1	2/21	48	20	96	128				
99	0	2/21	36	40	96	128				
100	C2	2/22	10	20	32	96				
101	F2	2/22	284	56	96	48				
102	C2	2/22	144	20	24	10				
103	F1	2/23	112	5	18	8				
104	0	2/23	384	48	96	40				
105	C1 F2	2/23	160	12	72	28				
106	0	2/23	24	6	10	2				
107	C2	2/23	70	6	14	3				
108	0	2/23	40	20	64	32				
109	F1	2/23	304	28	96	64				
110	0	2/23	40	5	24	12				
111	C1 2	2/25	192	56	80	28				
112	0	2/25	96	20	14	8				
113	V	2/25	144	14	128	48				
114	C1 F1	2/25	384	32	112	32				

(Continued on page 1)



TABLE IV—CONT'D

PATIENT	RECENT HISTORY OF CLINICAL INFLUENZA*	DATE OF SERUM	AVERAGE TITER OF INFLUENZA ANTIBODIES							
			PR8		JFE		WC		MF	
			AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
115	V, F1	2/25	256	6	96	24				
116	0	2/26	160	12	40	8				
117	0	2/27	40	6	16	12				
118	0	2/27	128	14	9	5				
119	0	2/27	40	7	56	16				
120	F1	2/27	240	16	15	14				
121	F1	2/27	28	6	48	16				
122	F2	2/27	144	12	7	2				
123	0	2/27	48	3	5	2				
124	0	2/27	12	2	10	8				
125	0	2/27	56	10	48	14				
126	0	2/27	14	3	80	14				
127	0	2/27	20	7	16	8				

\*C History of coryza and/or cough with minimum of fever and systemic symptoms  
F symptoms suggesting clinical influenza 1 onset between Nov 15 1945 and Jan 15 1946 2 onset after Jan 16 1946 V influenza A and B vaccine in November 1945 O no symptoms

laboratory workers from whom sera were obtained during the end of the second week in December and again during or after the first week in January. They had no symptoms of any illness during this interval or in the preceding few weeks. None of these individuals showed any rise in titer of antibodies for either the PR8 or the Lee virus. Low titers were obtained in all of these sera except for agglutinin inhibition titers greater than 64 obtained in both sera from one of this group with the Lee strain without correspondingly elevated complement fixation titers.

*Group VI Influenza Antibodies in Sera Obtained After the Epidemic*—In order to obtain some idea as to whether a retrospective history of respiratory infection during the epidemic might be reflected frequently in a high influenza antibody titer a few weeks later, single specimens of sera were obtained between Feb 16 and 23, 1946, from sixty-two individuals not included in any of the previous groups. Most of them were from young adult patients hospitalized for illnesses other than acute respiratory infections and others were from members of the hospital staff. Each person was questioned in detail concerning any illness that occurred during the preceding three months. All respiratory illnesses were then classified according to whether they most resembled colds or clinical influenza and whether they occurred before or after the middle of January. The occurrence of such infections as determined in this manner and the results of the tests for influenza antibody in their sera are listed in Table IV.

The outstanding finding in this group is the comparatively large proportion of sera showing elevated titers (greater than 64) of agglutinin inhibition of the PR8 strain without corresponding elevation of the complement fixation titers with the same strain. There were a few instances of elevated titers of agglutinin

inhibition of the Lee strain and in some of these sera the corresponding complement fixation titers were also higher than average. When this group is subdivided according to the recent history of respiratory infections, the numbers in each category are too small to permit any significant comparisons. However, except for the somewhat greater frequency of high agglutinin inhibition titers with both the PR8 and Lee strains in the person giving a recent history of clinical influenza, there seemed to be no striking predominance of significantly elevated titers in any group.

There were three persons in this group (Patients 81, 113, and 115) who had received an immunizing injection of influenza A and B vaccine in November 1945. One of them showed elevated titers by both tests against all the four strains of virus used. The other two showed elevated agglutinin inhibition titers with the PR8 and Lee strains without correspondingly elevated complement fixation titers. One of the latter also gave a history of clinical influenza during the height of prevalence of influenza B.

*Summary of the Serologic Findings*—The distribution of titers of PR8 and Lee antibodies in the seven groups that have been discussed is shown in Table V. The average titers for each of these groups are also shown. Groups I and IV\* stand out clearly from among the others. In Group I there was a similar distribution of titers in the lower range of both agglutinin inhibition and complement fixation of the PR8 strain in both acute and convalescent phase sera, whereas there was a shift in Lee antibodies from low titers in the acute phase to titers in the higher range in the convalescent phase sera. In accord with this distribution the average titers were essentially the same for acute and convalescent sera in the tests done with PR8 (though the actual numbers were higher for the average agglutinin inhibition titers than for the complement fixation titers), while the average anti-Lee titers were about twenty-fold greater during convalescence in the agglutinin inhibition tests and about thirty-five-fold greater in the complement fixation tests.

The reverse was true in Group IV. The number of cases in this group was small, but the distribution of anti-Lee antibodies was in the low range and the average titers were low and were the same by both tests in the acute and convalescent phase sera. The tests with PR8, on the other hand, gave low titers in the acute sera and high titers during convalescence. The average anti-PR8 titers were about ten times greater in the convalescent phase sera than in the acute phase sera done with either test.

In Groups IIA, III, and V the distribution of titers and the average titers were all similar to those found in the acute phase sera in Groups I and IV. In Groups IIA and V this was true for both the early and the late sera.

Groups IIB and VI are similar in several respects and stand out from the other groups. The sera in both of these groups were obtained after the date of the last proved case of influenza B. In each instance there was a smaller proportion of very low titers and more of the intermediate and the moderately elevated titers of agglutinin inhibition with PR8. The averages of these titers

\*The group designation corresponds to the one used in Table A and also to the one used in the previous section heading in the text.

TABLE V SUMMARY OF RESULTS OF SEROLOGIC TESTS WITH PR8 AND LEE STRAINS IN SEVERAL GROUPS OF PATIENTS

GROUP*	STRAIN	TEST†	NUMBER OF CASES	FIFTY†												AVERAGE‡
				4	8	16	32	64	128	256	512	1024	2048			
I Acute Convalescent	PR8	AI	23	2	3	4	8	2	1	1					36	
	PR8	AI	23	2	1	5	7	3	2						28	
IIA Acute Convalescent	PR8	AI	21	2	4	8	2	1	4						30	
	PR8	AI	21	2	1	6	4	2	3						29	
IIB Acute Convalescent	PR8	AI	8	0	0	1	1	4	1	1					75	
	PR8	AI	8	0	0	0	2	2	2	2					106	
III Acute	PR8	AI	20	3	1	3	6	2	1	1					36	
IV Acute Convalescent	PR8	AI	8	0	0	2	3	2	1						41	
	PR8	AI	13	0	0	0	0	1	1	7	2	1	1		416	
V Early Late	PR8	AI	11	3	1	2	2								13	
	AI	VI	11	1	2	2									13	
VI Acute	PR8	AI	62	0	1	1	9	16	16	11	5				109	
I Acute Convalescent	Lee	AI	23	6	8	4	1	0	0	11					18(11)	
	Lee	AI	23	1	0	3	3	6	2	3	3	1	1		234	
IIA Acute Convalescent	Lee	AI	21	6	3	3	3	2	2						21	
	Lee	AI	21	7	2	5	2	3	2						23	
IIB Acute Convalescent	Lee	AI	8	1	0	1	4	2							26	
	Lee	AI	8	1	1	1	3	2							26	
III Acute	Lee	AI	20	3	2	1	3	7	1						32	
IV Acute Convalescent	Lee	AI	8	2	1	1	0	2	14	0	0	14			196(23)	
	Lee	AI	13	1	2	2	4	3	0	1					97(28)	
V Early Late	Lee	AI	11	3	3	1	0	1	1	1					20	
	Lee	AI	11	3	4	1	1	1	1	1					22	
VI Acute	Lee	AI	62	2	7	12	8	13	18	1	1				63	

I	Acute	PR8	CF	23	6	6	5	6	11	13
IIA	Convalescent	PR8	CF	23	5	8	4	5	1	15
IIA	Acute	PR8	CF	21	5	4	3	7	1	15
IIA	Convalescent	PR8	CF	21	4	5	4	5	2	19
IIB	Acute	PR8	CF	8	1	2	1	4		17
IIB	Convalescent	PR8	CF	8	0	3	1	4		16
III	Acute	PR8	CF	20	6	6	7	1		8
IV	Acute	PR8	CF	8	3	1	0	3		16
IV	Convalescent	PR8	CF	13	0	0	0	1	3	14
V	Early	PR8	CF	11	3	2	3	3		13
V	Late	PR8	CF	11	2	3	5	1		11
VI	Acute	PR8	CF	62	8	13	18	17	6	16
I	Acute	Lee	CF	23	13	5	3	1	11	8(5)
I	Convalescent	Lee	CF	23	0	0	2	4	4	177
IIA	Acute	Lee	CF	21	7	4	6	3	1	12
IIA	Convalescent	Lee	CF	21	8	4	3	5	1	12
IIB	Acute	Lee	CF	8	3	2	2	1		9
IIB	Convalescent	Lee	CF	8	3	2	2	1		9
III	Acute	Lee	CF	20	8	2	5	3	1	15
IV	Acute	Lee	CF	8	1	1	3	0	1	52(15)
IV	Convalescent	Lee	CF	13	5	1	4	1	1	18(10)
V	Early	Lee	CF	11	3	2	3	3		14
V	Late	Lee	CF	11	3	2	4	2		12
VI	Acute	Lee	CF	62	10	12	18	12	6	21

I Patients with evidence of influenza B (Table I) II Cases of acute respiratory infection occurring prior to mid January without evidence of influenza A or B (Table II) Patients 1 to 14) II B cases similar to those in II but occurring after mid January (Table II)

† Inhibition of chicken cell agglutination CF complement fixation

‡ Each category includes intermediate titers up to next preceding one (e.g. 4 includes 1 or less 8 includes 5 etc.)

§ Corrected average (after excluding sera obtained on or after the seventh day or those indicated by †) shown in parentheses

|| Acute blood on eleventh day

¶ Further influenza B infection the values in the two cases are excluded in the corrected averages

were two to three times greater than in all groups except Group IV (convalescent sera from the patients with influenza A). The values for the convalescent phase sera in Group IIB were slightly but not significantly higher than for the acute phase sera. On the other hand, the distribution of the complement fixation titers was the same and the average of these titers obtained with the same virus in these two groups of cases was not elevated and corresponded to those observed in all other groups except in the convalescent phase sera in Group IV.

With respect to influenza B antibody, however, Groups IIB and VI differed. In the former, the distribution of titers was in the low range and the average titers in both the acute and convalescent phases were low and corresponded to those observed in Groups IIA, III, and V and in the acute phase sera in Group I. In Group VI, on the other hand, there was a greater proportion of intermediate and slightly elevated titers of Lee antibodies and the averages of these titers were two to three times those found in the remaining groups other than Group I (convalescent sera from patients with influenza B). In these respects the findings with the agglutinin inhibition and complement fixation tests were similar.

*Comparison of Results Obtained With the Lee Strain and With Two Epidemic Strains*—It will be seen from Tables I, II, and IV that, although there were some discrepancies in individual sera, the results obtained with the standard Lee strain and with WC and MF (the two strains of influenza B virus that were isolated early in the course of the epidemic) were very similar. The average titers of all the sera of these three groups that were tested with the three strains are shown in Table VI. Considering the rather small numbers involved, the average titers obtained in each group with the three strains were remarkably similar.

TABLE VI. COMPARISON OF AVERAGE TITERS OBTAINED WITH LEE STRAIN AND WITH TWO EPIDEMIC STRAINS IN THREE GROUPS OF PATIENTS

GROUP*		LEE			WC			MF		
		NUMBER OF CASES	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	NUMBER OF CASES	AGGLUTININ INHIBITION	COMPLEMENT FIXATION	NUMBER OF CASES	AGGLUTININ INHIBITION	COMPLEMENT FIXATION
I	Acute	22	11	5	18	4	5	12	4	4
	Convalescent	23	234	177	18	67	151	12	77	169
IIA	Acute	21	21	12	11	6	13	12	7	11
	Convalescent	21	23	12	11	6	14	12	7	12
VI		62	63	23	22	27	26	22	24	29

\*See footnote \* Table V

#### COMMENT

The serologic findings in Groups I and IV indicate quite clearly that both influenza B and influenza A occurred in Boston during the winter of 1945-1946. The dates of onset of illness in the patients of those two groups indicate that, for the most part, there were distinct and consecutive outbreaks. In this study

the proved cases of influenza B with one exception, had an onset between Dec 12 and Jan 3, the onset in the cases of serologically proved influenza A, again with one exception occurred between Jan 18 and Feb 3. Viruses giving characteristic reactions of influenza B were isolated from patients with acute cases during the former period,<sup>1</sup> but attempts to isolate viruses from two others with acute cases of influenza A were not successful.

The occurrence of both influenza B and A with a predominance of the former, was also reported in the corresponding epidemics in Australia<sup>4</sup> and Great Britain.<sup>5, 6</sup> In these countries too, there appeared to be consecutive outbreaks although there was somewhat more overlapping. Strains of both types of virus were isolated in Australia. Among the Australian cases there were two and possibly three instances of consecutive infections with influenza B and A in the same individuals, and two such cases are included among those reported here. Evidence was also obtained from complement fixation tests done on serum pools that influenza A occurred sometime after the influenza outbreak of December 1945, in New York State.<sup>13</sup> The isolation of influenza A virus in the Chicago area in December, 1945,<sup>17</sup> and serologic evidence that influenza A alone occurred in an outbreak at a boys' school thirty miles from Boston between Jan 9 and Feb 6<sup>18</sup> have also been recorded.

Symptomless infection associated with a significant rise in influenza virus antibodies was observed during this study in only one instance namely the influenza A infection that occurred in Patient 59 (Table III) following the attack of clinical influenza which had given rise to influenza B antibodies. Not enough observations were made to determine the incidence of such symptomless infections, but the findings in Group VI suggest that they were not infrequent with the B virus and those in Groups IIB and VI suggest that they may also have occurred with some type of A virus.

As for the remaining serologically proved cases of influenza B (Table I) and A (Table IV), the rise in antibodies followed a characteristic attack of clinical influenza in every instance except in one patient of each of these groups. A similar observation was made in the Australian cases.<sup>4</sup> The symptomatology varied in the cases that were not associated with an antibody rise both in those which occurred during the prevalence of influenza B and in those which occurred later when cases of influenza A were encountered. Some of those patients had symptoms of clinical influenza indistinguishable from those experienced by the patients showing definite antibody rises but a large proportion of them had symptoms that were more like those of the common cold or were predominantly gastrointestinal. Furthermore, in the patients who had two distinct infections one resembling clinical influenza and the other more like the common cold the antibody response seemed to be temporally related to the former.

The failure of certain patients with typical symptoms of influenza to show antibody rises to the epidemic strain even when they have low titers during the acute phase has been noted frequently by others. This has been observed even in patients with typical illness from whom a virus has been isolated<sup>14</sup> or who had been infected experimentally.<sup>19</sup>

The elevated titers of agglutinin inhibition of PR8 not accompanied by correspondingly elevated complement fixation titers are of some interest. They occurred in sera obtained after the middle of January from patients with clinical influenza who failed to show any significant antibody rise with the viruses used (Group IIB) and were also noted in the sera obtained from individuals picked at random late in February (Group VI). These may represent, in part, a response to infection with an influenza virus related to but not identical with PR8. Such a possibility is not inconsistent with the occurrence of typical rises of PR8 antibodies demonstrable by both agglutinin inhibition and complement fixation in other individuals, as noted in Table III.

The findings in Group VI suggest, in addition, that an appreciable proportion of the individuals in that group had had recent infection with influenza B, as evidenced by the elevated titers to those viruses demonstrable by both tests. The correlation of the elevated titers with the occurrence, time, and character of the infection as noted in the histories of these individuals was not very good. Some evidence was previously adduced to suggest that the WC and MF viruses were antigenically different from the Lee strain.<sup>12-15</sup> Although similar antibody responses to all three strains were observed in most patients, there were sera which showed definite discrepancies both in the present cases and in those reported from Needham.<sup>14</sup> Further observations<sup>15</sup> have since been made to support the view that WC and MF are more closely related antigenically to the Australian strain BON,<sup>20</sup> and similar strains have been identified both in Australia<sup>21</sup> and Great Britain during the 1945 epidemics in those countries.

#### SUMMARY AND CONCLUSIONS

Both influenza B and influenza A were prevalent in Boston during the winter of 1945-1946. Influenza B was responsible for the major outbreak that occurred in December, 1945, and no acute cases proved to be due to this virus were encountered after the first week in January. Cases of serologically proved influenza A were encountered almost entirely after the middle of January. Two cases were observed in which there was definite serologic evidence of consecutive infections first with influenza B and a few weeks later with influenza A. There was also some evidence of symptomless infections with viruses of both types.

Almost all of the patients in whose sera a rise in specific antibodies to one or another of the influenza viruses was demonstrated had respiratory and systemic symptoms which were characteristic of clinical influenza. Similar symptoms were noted during the same period in other individuals whose sera failed to show such a rise. A large proportion of the patients who failed to show a rise in influenzal antibodies, however, had an illness which was more like the common cold or had predominantly gastrointestinal symptoms.

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## INFLUENZA AND PNEUMONIA

### SEROLOGIC STUDIES DURING AND AFTER AN OUTBREAK OF INFLUENZA B

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IT IS generally assumed that deaths from influenza are usually associated with pneumonia, as seemed evident from clinical and pathological findings during the pandemic of 1918. Excess mortality rates during periods of epidemic prevalence of influenza, however, are credited as much to increases in deaths from other causes as they are to increases in deaths from influenza and pneumonia.<sup>1</sup> Evidence for a direct relation of influenza virus infection to the occurrence or severity of pneumonia in individual patients is rather scant.

This relationship of influenza virus infection to bacterial and other pneumonias has been a matter of interest in this laboratory for the past few years. Some serologic evidence for an association of influenza A infection with cases of pneumococcal and, particularly, of staphylococcal pneumonia was obtained during the 1940-1941 outbreak.<sup>2-4</sup> Presumptive evidence for the presence of influenza A in the lung of a patient with a fulminating case of staphylococcal pneumonia was obtained at that time.<sup>2-4</sup> Significantly elevated titers of influenza A antibodies also were demonstrated in cases of pneumonia of varied etiology which followed attacks of clinical influenza during the epidemic of 1943-1944.<sup>5</sup> In addition, influenza A virus was isolated from the lungs of five patients with fatal cases.<sup>6</sup> Two of the strains were from patients with the fulminating acute hemorrhagic and edematous type of staphylococcal pneumonia, a third was from a patient with lobar pneumonia in whom type 1 pneumococcus was obtained from the sputum, blood, and lungs, and cultures from the two remaining patients yielded no significant pathogenic bacteria and only a few alpha hemolytic streptococci from sputum and lungs. The onset of pneumonia occurred from one to three days after the onset of symptoms of clinical influenza in these five patients.

Other workers also have reported the isolation of influenza A virus from the lungs in acutely fatal cases of staphylococcal pneumonia, three such cases were reported from the 1936-1937 epidemic in England,<sup>7</sup> and one occurred in Philadelphia in January, 1939.<sup>8</sup> Various other types of lung changes were observed in patients with clinical influenza in the 1936-37 epidemic,<sup>7,9</sup> but the commonest lung lesion in proved cases of influenza A infection in that outbreak was considered to be a "bronchiolitis".<sup>9</sup>

The occurrence of pneumonia during epidemics of influenza B also has been reported, but in these outbreaks the evidence for the association of the virus with the individual cases of pneumonia is scant. Four fatal cases of pneu-

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pneumococcal lobar pneumonia and four other patients with pulmonary involvement were observed during an epidemic of influenza B in a mental institution in Minnesota.<sup>10</sup> The presence of influenza B infection during an epidemic of type 1 pneumococcal pneumonia in Northville N. Y. was demonstrated serologically.<sup>11</sup> In neither of these two outbreaks, however, was the relation of the influenza B virus infection established in any of the instances of pneumonia either serologically or by demonstration of the virus. Only one case has been reported prior to the epidemic of 1945 in which influenza B virus was isolated from the lung of a patient with a fatal case.<sup>1</sup> In this case as in most of those previously mentioned in which influenza A virus was yielded from the lungs there was a fulminating acute hemorrhagic and edematous type of *Staphylococcus aureus* pneumonia.

During a localized outbreak of influenza B which occurred in the Bahamas in 1945,<sup>12</sup> lobar pneumonia was recognized as a complication occurring from 0 to 14 days after the onset of clinical influenza in about 10 per cent of the cases. The relation of the virus was not established in any of the cases of pneumonia in this outbreak. Influenza B virus was isolated in Melbourne during the 1945 outbreak<sup>14</sup> from bronchial secretion obtained at autopsy from a patient with an acutely fatal case of staphylococcal pneumonia which began five days after the onset of influenza. This virus was also isolated in January 1945 from throat gargling obtained "from a nurse in Surrey with a severe form of influenzal pneumonia."<sup>11</sup>

The epidemic of influenza that occurred in Boston in December, 1945 offered an opportunity to make some serologic studies of influenza antibodies in cases of pneumonia that occurred during and shortly after the time when influenza B, and later influenza A infections were demonstrated in cases of clinical influenza.<sup>16</sup> The findings in these cases are reported here.

#### MATERIALS AND METHODS

*Choice of Patients*—All of the patients chosen for this study were admitted to the Medical Wards of the Boston City Hospital mostly during December 1945 and January, 1946. Detailed histories were obtained with respect to the pneumonia and to any antecedent acute respiratory illness in an attempt to determine the time of onset of these infections as nearly as possible.

Bacteriologic studies of blood and sputum were carried out during the acute illness in almost all of the cases. Blood for serologic tests was obtained during the acute febrile stage of the illness and again at intervals thereafter. Patients from whom samples of serum were available within the first week of the onset of respiratory illness but not later are excluded here. The findings in the sera of several such patients, including five with fatal cases, are given elsewhere.<sup>16</sup> No attempts were made to isolate viruses from these patients and autopsies were not obtained in any of the fatal cases.

*Clinical Findings*—The onset of pneumonia during or after a simple upper respiratory tract infection was usually marked by one or more of a number of manifestations: (1) the sudden occurrence of a brisk chill or of pleuritic pain

or both, (2) a recurrence of chills or chilly sensation, (3) the appearance of bloody or rusty sputum, (3) dyspnea, and (4) a change in the character and severity of the cough. The criteria for the classification of the upper respiratory tract infections were the same as those used in collateral studies of this outbreak reported elsewhere<sup>16, 17</sup>. The pneumonias were classified as either lobar or atypical (broncho-) pneumonia according to the clinical and x-ray findings. Some of the latter resembled characteristic cases of primary atypical ("viral") pneumonia.

*Serologic Tests*—The viruses used were the PR8 strain of influenza A and the Lee strain of influenza B\* and two strains WC and MF of influenza B isolated from uncomplicated cases of clinical influenza early in the course of this outbreak. The inhibition of chicken cell agglutination and the complement fixation tests were both carried out in every instance. The details of the methods used and the manner of recording the titers are the same as those used in other parallel studies<sup>17</sup>.

Tests for cold agglutinins were also carried out in all of these sera by the method usually employed in this laboratory<sup>18</sup>. The lowest final dilution of serum used in these tests was 1:10.

## RESULTS

A total of sixty-nine patients was available for study. They have been divided for convenience into three groups: those in whom a significant rise in influenza B antibodies was demonstrated, those whose initial serum showed significantly elevated titers of influenza B antibodies, and those in whom the serologic findings failed to reveal any evidence of recent influenza virus infection. The findings in each of these groups will be considered separately.

*Group I: Patients Whose Sera Showed a Significant Rise in Titer of Influenza B Antibodies*—There were eleven such patients, seven were women and four men and they ranged in age from 13 to 69 years. The relevant findings pertaining to the influenza and the pneumonia and the results of the serologic tests in these patients (1 to 11) are shown in the upper part of Table I. There was an illness which, from the history was considered to be clinical influenza in all but one of these cases. The first symptom of this illness began on November 30 in one patient and between December 18 and January 11 in the others. The onset of the pneumonia occurred on the same day in three cases, and one to ten days later in the others. In Patients 6 and 11 there were symptoms suggesting, respectively, an exacerbation of the original attack after five days and a second attack after six weeks.

The findings in the lungs were those of typical lobar pneumonia in two of these patients and cultures of the sputum yielded predominantly pneumococci in both. In all the others there was patchy consolidation. In Patient 1, the findings in the lungs closely resembled those of primary atypical pneumonia, sputum cultures yielded only alpha hemolytic streptococci. *Staph aureus* was the predominant organism in repeated cultures in four other patients and pneumococci predominated in the remaining four, with staphylococci being recovered in moderate

\*Both originally obtained from Dr. Thomas Francis, Jr., Ann Arbor, Mich.

numbers, particularly during convalescence from one of the latter patients. It is of interest that in Patient 1 who yielded no significant pathogens, and in the four patients in whom *Staph aureus* predominated pneumonia began either on the same day (three cases) or within two days of the onset of influenza.

There was slight to moderate leucocytosis with polymorphonuclear predominance during the febrile stage of the pneumonia in eight of these patients. In the other three the total counts averaged between 4 000 and 9 000, the lowest counts being obtained in one of the patients with staphylococcal pneumonia. Blood cultures were negative in all of the eleven patients and cold agglutinins could not be demonstrated in any of the sera. All but two of the patients were treated with sulfadiazine or penicillin or both, and the response to this treatment varied from fair to excellent. All recovered without complications.

The serologic findings in these cases were similar in every respect to those found in uncomplicated cases of influenza B which were studied at the same time<sup>16-17</sup>. The initial titers of influenza B antibodies were low in every subject except Patient 5, and in that patient the initial serum was obtained eight days after the onset of influenza. In every case including Patient 5, there was at least a fourfold and usually a much greater rise in titer of influenza B antibodies demonstrable by both the agglutinin inhibition and complement fixation tests. Similar results were obtained with the Lee strain and with the two recently isolated strains in the sera of four patients who were tested with all three strains.

None of these patients showed a significant rise in titer of antibodies to PR8. Agglutinin inhibition titers of 64 or higher were found in the sera of four of the patients and complement fixation titers of 32 or higher were obtained in the sera from these four patients only.

The time relationships between the influenza B antibody response and the onset of the infections in three patients are worthy of comment. In Patient 9 the initial titers of agglutinin inhibition of the influenza B viruses were all low. This serum was obtained thirteen days after what was considered to be the onset of clinical influenza. A significant rise in these antibodies was demonstrated in serum obtained five days later, or eight days after the onset of the pneumonia. Since rises in titers have usually been found in adults by the eighth day these findings suggest the possibility that the initial illness either was not influenza B or else it failed to elicit an antibody rise until after pneumonia began. The findings in Patient 10 were similar but in this case there were significantly elevated titers of PR8 antibodies in the initial serum which was obtained ten days after the onset of influenza and five days after what was considered to be the onset of pneumonia. The rise in B antibodies was demonstrated in serum obtained five days later. In Patient 11 there were two illnesses six weeks apart and from the history both were considered to be clinical influenza. The pneumonia began on the same day as the second attack and the rise in influenza B antibodies occurred between three and eleven days later.

*Group II Patients With Serologic Evidence of Recent Infection With Influenza B*—There were twenty-five patients in this group (12 to 36 in Table I). Thirteen were men and twelve were women and they ranged in age from 13 to 80 years. A history considered to be that of clinical influenza was obtained in



No	P	J <sub>a</sub> -J <sub>b</sub>	++	L <sub>1</sub> -L <sub>2</sub>	B	R/L	I <sup>-</sup>	0	S hem	I + S	+	1/-	I <sup>-</sup>	31-180
18	M	76	12/23	++	12/2-	L Rm	6	0	Pn 7	P	+++	2/13	1- 3	102 440
19	M	62	12/23	++	12/28	B R/L	16 13	0	Pn 3	S	+++	1/9	4 3	384 384
20	M	62	12/23	++	12/28	B R/L	16 13	0	Pn 3	S	+++	1/5	6 24	18 32
21	F	52	12/3 12/25	++	12/28	L I u	16 10	Pn 4	Pn 4, S au	P+S	+++	12/30	24 20	56 48
22	M	35	12/27	++	12/28	L L	13	Pn 7	Pn 7	P	+++	2/13	6 5	88 272
23	M	31	?	C	12/29	B Rm	8	0	S var f	0		1/14	32 10	128 80
24	F	36		0	12/30	I Rl	8	0	Pn 8	S	++	1/9	24 14	96 40
25	F	21	1/1	++	1/1	B R/Lu	18	S au	--	P+S	++	1/8	24 10	384 96
26	M	50		0	1/1	L Lhu	12 14	0	Pn 7	S	+++	1/4	3 2	192 192
27	M	56	1/1	++	1/4	I Rl	13	Pn 5	Pn 17 Pn 5	P+S	+++	1/8	4 2	512 320
28	F	21	12/27	++	1/5	L Rml	21 24	0	--	S	+++	1/4	16 3	288 54
29	F	50	12/25	++	1/6	B Rl	21	0	--	P+S	+++	1/8	8 2	336 60
30	F	54	12/27	+++	1/7	B R/H	12	0	Pn 22 Pn 10	S	++	1/4	2 2	88 298
31	M	35	12/29	+	1/9	L Rl	18	0	Pn 1	S	++	1/12	2 2	128 80
32	M	13	1/4	++	1/12	B Rl	14 19	0	S var f	P	++	1/18	64 16	2048 128
33	M	63	1/12	++	1/12	B Lal	10 4	0	S au	P+S	++	1/18	283 13	2048 160
34	F	30	1/11	+	1/19	I Rml	16	0	Pn 12 Pn 1	P+S	+++	1/12	64 56	1536 640
35	F	65	1/5	C	1/19	I Rml	22 13	0	Pn 1	P+S	++	1/19	4 12	768 112
36	M	80	?	?	B Blat	13	0	--	S	+++	1/16	3 1	4 12	1024 160

Table 1. Clinical and laboratory findings in patients with influenza A and B. The table shows the number of patients with each finding, the number of patients with each finding who were also in the influenza A group, and the number of patients with each finding who were also in the influenza B group. The findings are listed in the first column, and the numbers are listed in the second, third, and fourth columns.

Finding	No. of patients	No. of patients (influenza A)	No. of patients (influenza B)
Malaise	10	6	4
Fatigue	10	6	4
Headache	10	6	4
Myalgia	10	6	4
Arthralgia	10	6	4
Pharyngitis	10	6	4
Hoarseness	10	6	4
Cough	10	6	4
Sputum	10	6	4
Leukopenia	10	6	4
Thrombocytopenia	10	6	4
Neutrophilia	10	6	4
Eosinophilia	10	6	4
Monocytosis	10	6	4
Lymphocytosis	10	6	4
Atypical lymphocytes	10	6	4
Antinuclear antibodies	10	6	4
Rheumatoid factor	10	6	4
Complement	10	6	4
Anticardiolipin antibodies	10	6	4
Anticardiolipin antibodies (IgG)	10	6	4
Anticardiolipin antibodies (IgM)	10	6	4
Anticardiolipin antibodies (IgA)	10	6	4
Anticardiolipin antibodies (IgE)	10	6	4
Anticardiolipin antibodies (IgG, IgM, IgA, IgE)	10	6	4

nineteen of these patients, including one who had two similar attacks three weeks apart, two others had an illness which was more like a common cold, while no history of an antecedent illness distinguishable from pneumonia could be elicited in the four remaining patients. The onset of these illnesses occurred between December 3 and January 12, and in all but three instances they began between December 15 and January 5.

The interval between the onset of influenza or cold and pneumonia was seven days or less in twelve cases and from eight to fourteen days in eight others. The pulmonary lesion in thirteen patients was that of typical lobar pneumonia involving a single lobe or two contiguous lobes. Pneumococci predominated in cultures of the sputum in eleven of these patients and were also grown from the blood of four of them, in one of the latter the sputum cultures yielded predominantly *Staph aureus* during convalescence. Cultures of sputum in one instance yielded only alpha hemolytic streptococci, and none were made in one case.

The remaining twelve patients had atypical pulmonary lesions which involved chiefly a single lobe in five, two contiguous lobes in two, and both lungs (mostly the lower portions) in the other five. *Staph aureus* was obtained from a blood culture of one of these patients and was predominant in the sputum cultures of two others, including one which yielded pneumococci as well, beta hemolytic streptococci were the predominant organisms in the sputum in two patients and pneumococci predominated in two others. Cultures in the remaining five cases were either inadequate or yielded no significant pathogens.

The leucocyte counts were elevated during the febrile course in most of the patients with atypical pulmonary lesions and were similar in those with lobar and atypical pneumonia. Total counts of 8,000 or lower were obtained some time during the febrile course in two of the former and in three of the latter. In the others the total counts ranged between 12,000 and 40,000, with polynuclears predominating in every instance. None of the patients in this group showed elevated titers or rises in titers of cold agglutinins.

All but one of the patients in this group were treated with sulfadiazine, penicillin, or both and responded favorably to this treatment. They all recovered and became essentially afebrile within twenty-four to seventy-two hours after the treatment was started.

The initial serums were obtained in almost all of these cases between the sixth and fourteen day after the onset of the initial illness and within the first week after the onset of the pneumonia. The titers of influenza B antibodies in these initial sera were significantly elevated and about equally so in both tests and for each of the B strains used. There were six patients in whom there were suggestive or definite rises in these titers on repeated tests with one or more of the B strains. The initial serum in four of these six patients was obtained on or before the seventh day after the onset of clinical influenza. In three other patients the later sera showed a definite drop from high titers, the initial serum was obtained on the twelfth day in one and on the thirteenth day in the second, while the third patient had two distinct illnesses which began twenty-seven and five days, respectively, before the first blood was taken. In the latter case a suggestive drop was noted in the titer of the second blood obtained ten days after the first one.

No significantly elevated titers or rises in titers of antibodies to the PR8 strain were obtained in this group except in Patient 31. In this patient a slightly greater than fourfold rise was elicited by the agglutinin inhibition tests, while the complement fixation tests with the same virus yield low titers and no rise.

*Group III Patients Lacking Serologic Evidence of Influenza Virus Infection*—The relevant findings in thirty-three such patients are listed in Table II. The clinical features of this group resembled those of the two previous groups in some respects but showed some important points of difference. The age distribution, for example, was similar but the sex distribution in the present cases differed from that of the other groups and was more like that usually found in acute bacterial pneumonias in adults. The patients ranged in ages from 14 to 75 years and there were twice as many men as women.

A larger proportion of the antecedent illnesses in this group were simple colds. Thus, of twenty-nine patients from whom a history of antecedent illness distinct from the pneumonia could be elicited, that illness was classified as clinical influenza in twelve as a cold in twelve, and as a cold followed five to twenty-six days later by clinical influenza in five. The interval between the onset of the illness that was characterized as clinical influenza and the onset of pneumonia was two days or less in twelve cases and five to ten days in five cases. The interval between the onset of the cold and that of the pneumonia was less than two days in only two patients but five to ten days in six and eleven to twenty-eight days in eight.

The pulmonary lesion was classified as typical lobar pneumonia in nineteen patients, involved a single lobe in fourteen and two contiguous lobes in the other five. The predominant organism in the sputum was *Staph aureus* in one of these patients and the same organism was obtained from the blood culture of that patient. pneumococci predominated in the sputum of seventeen including one with a positive blood culture, and the bacteriology was inadequate in one case.

The remaining fourteen patients had atypical pulmonary lesions involving mostly the lower portions of the lung—one side in eight and bilaterally in six. The predominant organisms varied more than among the lobar pneumonias. *Staph aureus*, beta hemolytic streptococci, pneumococci, and Friedlander's bacilli each were found in two or three cases alone or in combination with one of the others. A positive blood culture for hemolytic streptococci was obtained in one case. In two patients no adequate bacteriological studies were made and in three others the sputum cultures yielded predominantly alpha hemolytic streptococci but no significant pathogens.

Cold agglutinins were demonstrated (a rise from  $< 10$  to 80) in one of the latter but in none of the other thirty-two patients in this group. The leucocyte counts during the febrile period in this group were similar to those found in the other groups. Total counts of 5,000 or less were obtained in three cases between 6,000 and 10,000 in six and from 11,000 to 28,000 in the others. Sulfadiazine, penicillin or both were used in the treatment of all but three of these patients with good effects in most instances. There were two deaths in this group, one a 75-year-old patient with pneumococcal pneumonia complicated by congestive heart failure (Patient 61) and the other a 65-year-old patient with Friedlander's pneumonia.



TABLE II FINDINGS IN CASES OF PNEUMONIA LACKING SEROLOGIC EVIDENCE OF INFLUENZA B INFECTION

			SUBJUNT FEATURES OF INFLUENZA										AVERAGE TITER OF INFLUENZA ANTIBODIES									
PATIENT	SEX	AGE	CLINICAL INFLUENZA		DATE OF ONSET	TYPE OF LESION	BACTERIOLOGY			RESPONSE	DATE OF SERUM	PR8		III		WC		MP				
			DATE OF ONSET	SEVERITY			LOBES INVOLVED	WHITE BLOOD COUNT	BLOOD			SPUTUM*	ANTIBACTERIAL THERAPY	AGGLUTINATION INHIBITION	COMPLEMENT FIXATION	AGGLUTINATION INHIBITION	COMPLEMENT FIXATION	AGGLUTINATION INHIBITION	COMPLEMENT FIXATION			
37	M	51	12/1	C	12/9	B	RI	918	0	S au, II inf	S	+	12/20	8	3	6	3	3	3	3		
			12/9	++									1/2	8	3	6	3	3	3	3		
38	M	41	12/10	I	12/10	I	Rum	12	0	Pn 20, II inf	P	+	12/17	3	5	3	3	3	3	3		
39	F	41	12/13	C	12/12	L	RI	26	0	Pn 15, S au	P	++	12/19	6	10	8	6	5	5	5		
													12/26	8	7	8	7	5	5	5		
40	M	38	?	C	12/12	L	RI	6	0	Pn 1	P	+++	12/24	12	1	10	10	10	10	10		
													1/3	12	1	10	10	10	10	10		
41	M	15	12/4	C	12/15	L	Ll	22	0	Pn 2	P+S	+++	12/20	8	5	3	3	3	3	3		
			12/14	++									12/26	6	5	3	3	3	3	3		
42	F	14	12/19	C	12/20	B	Bilat	8	0	S var, S au	P+S	+	12/26	24	10	24	8	11	11	11		
													1/2	24	3	24	3	5	5	5		
43	M	54	12/20	++	12/20	B	Bilat	215	0	--	S	0	12/28	16	3	8	2	2	2	2		
													1/1	24	2	6	2	2	2	2		
44	M	60	12/2	C	12/21	L	RI	86	0	Pn 8	P	++	12/26	6	12	32	14	20	12	11		
													1/1	6	6	32	9	16	18	12		
45	M	48	12/10	++	12/19	L	Rml	2813	0	Pn 9	P	++	12/26	4	1	1	24	16	18	12		
													1/5	24	3	1	20	16	18	12		
46	M	34	12/20	+++	12/26	L	Rum	2015	S au	S au	P+S	++	12/26	24	3	7	6	6	12	10		
													1/3	32	3	3	3	3	12	8		
47	M	54	12/25	++	12/26	L	Ll	18	0	Pn 12	P+S	++	1/2	16	6	3	5	7	12	10		
													1/9	16	1	7	5	10	12	12		
48	F	35	12/1	C	12/28	L	Rml	11	0	Pn 8	S	+++	12/28	24	44	40	18	32	36	56		
			12/27	++									1/5	32	55	80	56	32	36	56		
49	F	47	12/13	C	12/27	B	RI	11	--	--	0		12/28	8	4	3	2	10	32	56		
													1/3	8	4	3	2	10	32	56		
50	M	37	12/22	C	12/27	B	RI	27	S hem	S hem	P	+	1/3	155	8	8	2	3	3	3		
													1/8	157	1	16	2	3	3	3		
51	M	60		0	12/29	B	Ll	6	0	Pn 21	S	++	1/3	1	2	3	2	3	3	3		
													1/10	6	2	3	2	3	3	3		



determinations were drawn immediately prior to initiation of dietary deficiency, just before infection, and two, five, ten, fourteen, twenty one, twenty eight and forty two days after infection. Twenty eight days after infection all animals were injected subcutaneously with *S. typhimurium* O antigen. The immune response was determined two weeks later. Antibody titers and electrophoretic distributions of plasma proteins were measured concurrently on the twenty eighth and forty second postinfection days. The experiment was terminated on the forty second postinfection day, forty eight days after protein deficiency was started and fifty eight days after the experiment began.

Young, growing, male, Sherman strain rats weighing 60 to 70 grams were used. In this study, four experimental groups were established as follows:

	WELL NOURISHED	PROTEIN DEFICIENT	TOTAL
Infected	20	18	
Noninfected	10	12	
	<hr/> 30	<hr/> 30	60

The animals were isolated in an air conditioned room and kept in individual wire cages on wide meshed wire bottoms. The diets used had the following composition:

#### DIET COMPOSITION PER 100 GM. RATION

FOOD	ADEQUATE RATION	PROTEIN DEFICIENT RATION
Casein	18 Gm	2 Gm
Sucrose	73 Gm	89 Gm
Mazola	5 Gm	5 Gm
Phillips and Hart salts IV <sup>20</sup>	4 Gm	4 Gm
Cystine	0.2 Gm	0.2 Gm
Thiurine chloride	200 gamma	400 gamma
Pyridoxine	200 gamma	400 gamma
Riboflavin	400 gamma	800 gamma
Niacin	25 mg	50 mg
Calcium pantothenate	15 mg	30 mg
Choline chloride	100 mg	200 mg
Inositol	100 mg	200 mg
Biotin	50 gamma	100 gamma

Haliver oil was fed in 0.1 cc amounts biweekly, water was offered ad libitum. Diets were isocaloric, providing 4.1 calories per gram ration. The 18 per cent casein diet afforded 155 to 368 mg nitrogen per twenty four hours, depending upon consumption, the 2 per cent casein diet, 19 to 39 mg nitrogen per twenty four hours. Twenty four hour food consumption was recorded biweekly.

The stock culture of the infecting organism, *S. typhimurium* (*Bacillus aertrycke*), was originally isolated from a mouse epizootic and subsequently maintained in a frozen state on an agar slant.\* This organism was characterized by smooth, pearly white, small, elevated colonies which produced acid and gas with mannite, dulcitol, maltose, dextrose, arabinose, inositol, sorbitol, and rhamnose and in Russell double sugar media, lead acetate was reduced, no reaction occurred with lactose, sucrose, or xylose. Subcultures at 37° C in tryptic digest broth were made sequentially at twenty four, twenty four, eighteen, and six hours. Diluted aliquots of the final, young, six hour subcultures were injected. In the preliminary studies a 10<sup>-3</sup> (1,000) dilution was found to provide a 50 per cent lethal intraperitoneal inoculum. In this experiment, 1 ml of a similar dilution containing 300,000 viable cells per milliliter was used in an endeavor to provide a 100 per cent infection rate without subsequent mortality.

At previously noted intervals, 1 ml of blood was obtained by heart puncture under light ether anesthesia. Sterile technique was used. An initial 0.1 ml of the sterile sample was placed in blood culture tubes containing 5 ml freshly prepared tryptic digest broth. The remainder of the sample, placed in small tubes containing 0.2 mg dried heparin, sufficed for all except electrophoretic determinations.

\*We are indebted to Dr. John Enders for the stock culture used.

on January 25, February 1, and February 2, respectively. The onset of the pneumonia was on February 12 in the first, February 6 in the second, and February 2 in the third. No significant bacterial pathogen was obtained in the first case, clinically that case resembled primary atypical pneumonia. The other two were typical cases of pneumococcal lobar pneumonia due to type 5 and type 4 pneumococcus, respectively.

Another interesting feature of the present cases was the difficulty sometimes encountered in differentiating between the onset of the influenza and that of the pneumonia. Indeed in some of the cases the onsets, at least from a clinical point of view, occurred almost simultaneously. In the others varying intervals elapsed before the pneumonia began but these intervals were usually shorter after illnesses that were characteristic of clinical influenza than they were after illnesses that resembled the common cold. That seemed to be true irrespective of whether or not the clinical influenza was associated with an antibody response to the virus.

The significance of the findings of evidence of influenza virus infection with respect to the occurrence, incidence, or severity of bacterial and other pneumonias is by no means clear. The findings in some cases suggest that the pulmonary lesion is due primarily, if not entirely to the virus but such cases are rare. In most of the cases, the character of the pulmonary lesion, the clinical course of the disease, and the response to antibacterial therapy is characteristic of infection with the predominant bacteria found in the sputum or lungs and does not seem to be markedly influenced by the antecedent influenza virus infection. Some cases, particularly those in which hemolytic *Staph aureus* is the predominant or only bacterial invader, may be exceptions.

The situation may be different under other conditions, such as those which prevailed during the influenza pandemic of 1918. The nature of the primary etiologic agent of that pandemic is not known but the incidence and severity of the complicating pneumonias at that time were different from those encountered during any of the epidemics which were known to be caused by varieties of influenza A or B. A close resemblance however was demonstrated between the staphylococcal pneumonias which occurred during the influenza A outbreak of 1940-1941 and the pulmonary complications that occurred in certain circumstances during the pandemic.<sup>3, 4</sup> There are very little data available from which one may determine whether the nature of the virus or of the bacteria or the proper combinations of virus and bacteria were responsible for some of the varieties of pneumonia that were encountered during that great pandemic.

#### SUMMARY AND CONCLUSIONS

Some of the relevant clinical findings in sixty-nine cases of bacterial and other pneumonias that were studied during and shortly after the epidemic of influenza B which occurred in Boston in December 1945, are presented. Serologic tests for antibodies to influenza A and B virus including two epidemic strains of the latter were carried out in sera obtained from these patients during and after the acute phase of the pneumonia.

About one half of the cases that occurred during the period of epidemic prevalence of influenza B irrespective of the clinical character of the pneumonia

of the bacteriologic findings, yielded serologic evidence of infection with influenza B virus. Almost all of the patients from whom serologic evidence of influenza B infection was obtained had an illness which was characteristic of clinical influenza, that illness began either at about the same time as the pneumonia or within a few days previous to the onset of pneumonia.

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# NUTRITIONAL STATUS AND INFECTION RESPONSE

## I. ELECTROPHORETIC, CIRCULATING PLASMA PROTEIN, HEMATOLOGIC, HEMATOPOIETIC, AND IMMUNOLOGIC RESPONSES TO *SALMONELLA* *TYPHIMURIUM* (*BACILLUS AFRTRYCKE*) INFECTION IN THE PROTEIN DEFICIENT RAT

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### INTRODUCTION

THE relationship between hunger, famine, and infection<sup>1</sup> has become more conspicuous by reiteration than by demonstration. Each newly isolated nutritional factor has been indicted for some inadequacy of infectious disease response.<sup>2</sup> In recent years, protein nutrition, in particular, has acquired considerable prominence in this regard. It has been inferred that protein inanition would increase susceptibility or decrease resistance to infectious disease by altering protective responses.<sup>3-4</sup> Clinical observations<sup>5-7</sup> and specific laboratory investigations<sup>8-16</sup> have somewhat implemented this hypothesis.

In general, laboratory studies pertaining to response to infection associated with protein deficiency have measured phagocytic indices,<sup>12-13</sup> humoral antibody titers produced by a nonpathogenic antigen,<sup>10-12, 14-15</sup> or a lethal end point produced by a virulent, viable pathogen.<sup>8-9, 11-16</sup> Concomitant studies of variations in specific physiologic responses to diet and infection have been rather limited.<sup>17-19</sup> The response of an organism to infection under any imposed condition is characterized by many adaptative phenomena. To further test the implications of protein deficiency and infection observations of several simultaneous physiologic adaptations to controlled dietary deficiency and specific infection are necessary. To this end, data are presented indicating growth, food consumption, concentration, total circulating and electrophoretic distribution of plasma protein, and bacteriologic, immunologic and hematologic studies in control and *Salmonella* infected rats, both well nourished and protein deficient.

### METHOD

After an observation period on adequate or protein deficient diets, animals were infected with virulent *Salmonella typhimurium*. Controls with regard to diet and infection were maintained. Preliminary experiments involving sizable groups of rats were performed to determine the 50 per cent lethal dose of viable pathogen, the incidence and duration of bacteremia, the alteration in leucocyte, hemoglobin and plasma protein concentrations associated with infection and adequate or deficient diet and the general trend of antibody titers. These studies were done before and one, three, seven, fourteen, twenty one, and twenty eight days after infection. The more extensive study comprising this report was based upon the unreported data of these preliminary experiments. In this study, blood samples for the various

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determinations were drawn immediately prior to initiation of dietary deficiency, just before infection, and two, five, ten, fourteen, twenty one, twenty eight and forty two days after infection. Twenty eight days after infection all animals were injected subcutaneously with *S. typhimurium* O antigen. The immune response was determined two weeks later. Antibody titers and electrophoretic distributions of plasma proteins were measured concurrently on the twenty eighth and forty second postinfection days. The experiment was terminated on the forty second postinfection day, forty eight days after protein deficiency was started and fifty eight days after the experiment began.

Young, growing, male, Sherman strain rats weighing 60 to 70 grams were used. In this study, four experimental groups were established as follows:

	WELL NOURISHED	PROTEIN DEFICIENT	TOTAL
Infected	20	18	
Noninfected	10	12	
	30	30	60

The animals were isolated in an air conditioned room and kept in individual wire cages on wide meshed wire bottoms. The diets used had the following composition:

#### DIET COMPOSITION PER 100 GM RATION

FOOD	ADEQUATE RATION	PROTEIN DEFICIENT RATION
Casein	18 Gm	2 Gm
Sucrose	73 Gm	89 Gm
Mazola	5 Gm	5 Gm
Phillips and Hart salts IV <sup>20</sup>	4 Gm	4 Gm
Cystine	0.2 Gm	0.2 Gm
Threonine chloride	200 gamma	400 gamma
Pyridoxine	200 gamma	400 gamma
Riboflavin	400 gamma	800 gamma
Niacin	25 mg	50 mg
Calcium pantothenate	15 mg	30 mg
Choline chloride	100 mg	200 mg
Inositol	100 mg	200 mg
Biotin	50 gamma	100 gamma

Haliver oil was fed in 0.1 cc amounts biweekly, water was offered ad libitum. Diets were isocaloric, providing 4.1 calories per gram ration. The 18 per cent casein diet afforded 155 to 368 mg nitrogen per twenty four hours, depending upon consumption, the 2 per cent casein diet, 19 to 39 mg nitrogen per twenty four hours. Twenty four hour food consumption was recorded biweekly.

The stock culture of the infecting organism, *S. typhimurium* (*Bacillus aertrycke*), was originally isolated from a mouse epizootic and subsequently maintained in a frozen state on an agar slant\*. This organism was characterized by smooth, pearly white, small, elevated colonies which produced acid and gas with mannite, dulcitate, maltose, dextrose, arabinose, inositol, sorbitol, and rhamnose and in Russell double sugar media, lead acetate was reduced, no reaction occurred with lactose, sucrose, or xylose. Subcultures at 37° C in tryptic digest broth were made sequentially at twenty four, twenty four, eighteen, and six hours. Diluted aliquots of the final, young, six hour subcultures were injected. In the preliminary studies a 10<sup>-3</sup> (1:1,000) dilution was found to provide a 50 per cent lethal intraperitoneal inoculum. In this experiment, 1 ml of a similar dilution containing 300,000 viable cells per milliliter was used in an endeavor to provide a 100 per cent infection rate without subsequent mortality.

At previously noted intervals, 1 ml of blood was obtained by heart puncture under light ether anesthesia. Sterile technique was used. An initial 0.1 ml of the sterile sample was placed in blood culture tubes containing 5 ml freshly prepared tryptic digest broth. The remainder of the sample, placed in small tubes containing 0.2 mg dried heparin, sufficed for all except electrophoretic determinations.

\*We are indebted to Dr. John Enders for the stock culture used.

Blood cultures were incubated for twenty four hours at 37° C. Positive growths were plated out on eosin methylene blue and blood agar plates, the small violet and small pearly white colonies respectively, of gram negative bacilli were appropriately identified. All negative cultures were kept five to seven days before being discarded.

Hemoglobin (oxyhemoglobin) was determined in the Klett Summerson photoelectric colorimeter, using a 540 mμ filter, and the relative cell volume was calculated from it.<sup>21</sup> Total plasma protein concentration was determined gravimetrically by the copper sulfate method, using the formula: Total protein (gm/100 cc) =  $389.6 (G_p - 100.9)^2$ . Total leucocyte and differential counts were done by the usual methods. Blood volume partition studies, using Evan's blue dye T 1824 and a modified single sample photoelectric microcolorimetric technique,<sup>1</sup> were done concurrently with the concentration determinations at several intervals. Total circulating hemoglobin and total circulating protein were readily calculated and adjusted to unit values (grams per unit blood or plasma volume). These unit values were derived by adjusting total volumes to a unit of surface area (milliliters per 100 cm<sup>2</sup>).<sup>22</sup> Surface area was calculated according to the formula:  $SA (cm^2) = 12.54 (weight)^{0.6024}$ .

Humoral group and type specific antibody titers were determined by a standard two fold eight tube serum dilution agglutination method. Five tenths milliliter of a 1:10 plasma mixture and 0.5 ml of the appropriate antigen were used in the first tube. The formalin treated, diluted suspension for floccular agglutination (H or flagellar antigen) and the alcohol treated, diluted suspension for granular agglutination (O or somatic antigen) were prepared from the original *S. typhimurium* inoculum by standard Massachusetts State Laboratory procedures.

Several animals of each group were sacrificed by heart puncture on the twenty eighth postinfection day, at the time of subcutaneous injection of O antigen in the remaining rats. Sera from the sacrificed animals were pooled by group for electrophoretic analyses. Electrophoreses of the sera were carried out in a modified Fishel's apparatus.\* Three to five milliliters of serum were diluted to 12 ml giving approximately 2 per cent protein concentration and subsequently dialyzed against sodium diethyl barbiturate buffer of pH 8.6 and 0.1 ionic strength. A 10 ml cell was used and runs of 7,200 seconds were made at 1 to 2° C and potential gradients of 5 to 6 V per centimeter. Photographs of schlieren patterns obtained with a cylindrical lens at an angle of 45°, were made and enlarged 1.5 by projection. Mobilities were calculated from descending boundary patterns. Relative proportions of the various plasma components were determined by planimetry of Gauss curve resolved areas. The reported compositional data represent an average derived from both ascending and descending boundary patterns, excluding the delta and epsilon boundary anomalies.

At time of death, bone marrow imprints<sup>26</sup> were obtained from the distal diaphysial third of the left femur, eight to ten consecutive imprints were made on each of three coverslips. A modified May Grunwald Giemsa stain was used. Each of one hundred adjacent cells derived from a well defined peripheral area of five consecutive imprints was identified and tabulated. Cytologic classification followed that of Strasney and Higgins<sup>6</sup> but megakaryocyte, myeloblast, promyelocyte, myelocyte, metamyelocyte, granulocyte, lymphoblast, lymphocyte, plasma cell, reticulum cell, erythroblast, pronormoblast, normoblast and unclassified cells were enumerated. To simplify evaluation these were grouped as immature myeloid forms, mature granulocytes, erythroblasts, normoblasts, lymphocytes, and other miscellaneous cells. Total marrow cellularity was not estimated.

## RESULTS

Results are summarized in Tables I through VI and Figs 1 through 3.

### Growth —

### DISCUSSION

**Caloric and Nitrogen Consumption** Weight increments of 3 to 5 Gm per day on the initial control diet represented an adequate growth rate for rats of

\*According to the technique of the Department of Physical Chemistry.<sup>23</sup>



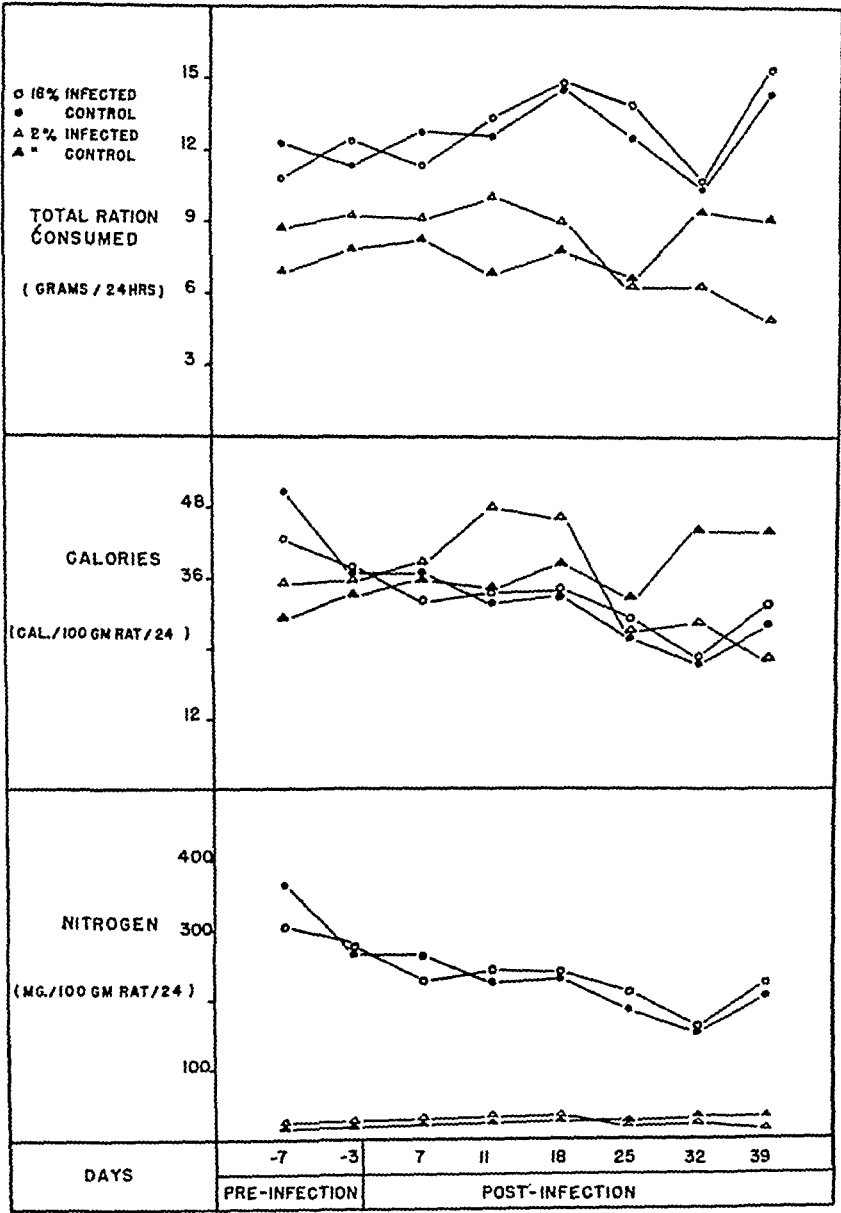


Fig 1—Relative average food consumption of control and infected rats on 18 per cent and 2 per cent protein diets. Total ration consumed as measured over a twenty-four hour period is compared with caloric and nitrogen intakes adjusted to a unit of body size

this age, sex, and strain, and for this diet. Progressive weight loss, ultimately amounting to 20 to 25 per cent of the starting body weight, attended protein deficiency. Growth responses to the 18 per cent and 2 per cent casein diets are indicated in Fig 1. Total food consumption per twenty-four hours per rat was greater in the well-nourished than in the deficient animals (10 to 15 Gm per twenty-four hours versus 6 to 10 Gm per twenty-four hours). The relative

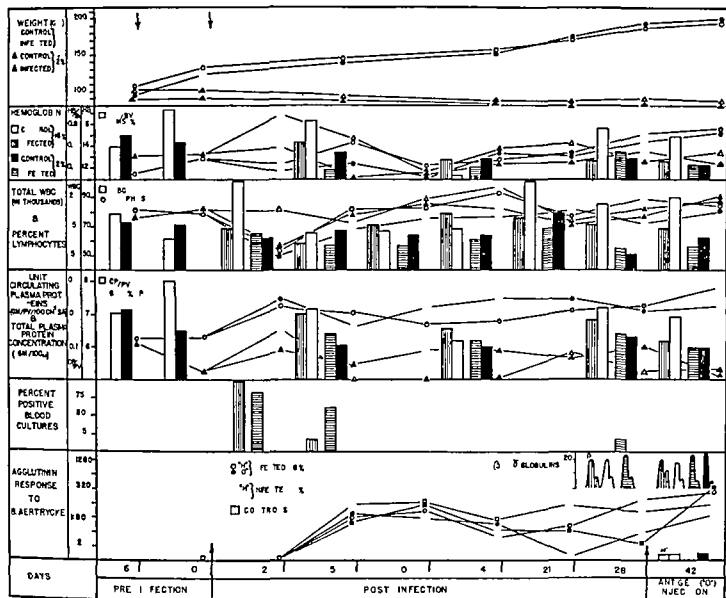


Fig —Summary of the blood and bacteriologic findings in control or *Salmonella* infected rats on 18 per cent or 2 per cent protein rations

caloric consumption per 100 Gm rat per twenty four hours however, was similar in both groups. During some phases of the study the protein deficient animals appeared to consume more calories per 100 Gm rat than did the well nourished animals. Irrespective of infection well nourished animals consumed 155 to 368 mg N per 100 Gm rat per twenty four hours and deficient animals 19 to 39 mg N per 100 Gm rat per twenty four hours (Fig 1). The similarity of nitrogen consumption in the infected and noninfected animals and the lack of striking differences (irrespective of dietary protein level) in caloric intakes per 100 Gm rat per twenty four hours serve in lieu of pair fed nutrition control data. Nitrogen consumption of the animals receiving an adequate diet appeared to be inversely proportional to growth and unaltered by infection. Nitrogen consumption of the deficient animals was maintained at a relatively constant level despite both infection and progressive weight loss.

#### Blood Studies —

**Hemoglobin** Hemoglobin concentration values in grams per 100 ml for rats of this strain and sex range from  $12.20 \pm 0.9$  to  $15.1 \pm 0.3^2$  and are similar to values reported for other strains. Hemoglobin concentrations of infected and noninfected animals followed similar trends. Differences between

TABLE I AVERAGE CONCENTRATION VARIATIONS

	PREINFECTION PERIOD			DURATION OF							
	INITIAL (10 DAYS)	DEFICIENCY (6 DAYS)		48 HOURS			5 DAYS			10	
Dietary level	18%	18%	2%	18%	18%	2%	18%	18%	2%	18%	18%
Infectious status	C	C	C	I	C	I	I	C	I	C	C
Number of animals†	6	3	3	5	2	5	5	2	5	2	2
Hemoglobin (Gm/100 cc)	12.4	13.1	13.1	11.9	12.8	14.0	12.9	14.4	10.6	14.7	11.0
		(2)‡			(1)		(3)				12.3
Total WBC/mm <sup>3</sup> (in thousands)	17.1	11.2	15.5	14.7	36.4	12.3	9.1	12.4	8.8	13.6	15.2
					(1)		(4)				13.2
Per cent polymorphonuclear leucocytes	19	18	16	48	39	44	29	20	17	26	21
					(1)						15
Per cent lymphocytes	81	81	83	50	53	54	69	83	82	73	77
					(1)						84
Total plasma protein (Gm/100 cc)	6.19	6.26	5.32	7.40	7.24	5.85	6.73	7.05	5.57	4.98	7.19
					6.43						6.74

C control I infected

\*These data represent a series of longitudinal determinations within specific groups. They involved re

†Number of animals contributing to each mean is small. Standard deviation of hemoglobin and total pro

‡Numbers in parentheses represent number of animals in group when that figure differs from number in

TABLE II AVERAGE CIRCULATING PROTEIN AND

	PREINFECTION PERIOD			5 DAYS			
	INITIAL (10 DAYS)	DEFICIENCY (6 DAYS)					
Dietary level	18%	18%	2%	18%	18%	2%	18%
Infectious status	0	0	0	I	C	I	C
Number of animals†	6	3	3	2	2	2	2
Surface area (SA = cm <sup>2</sup> )	203.5	225.0	192.0	270.9	265.1	182.4	171.8
Plasma protein concentration (TP = Gm/100 cc)	6.18	6.26	5.29	6.69	7.05	5.43	4.49
Total plasma volume (PV <sub>t</sub> = cc)	6.81	10.08	5.63	8.34	8.98	4.63	3.71
Unit plasma volume (PV <sub>u</sub> = cc/100 cm <sup>2</sup> SA)	3.32	4.44	2.94	3.04	3.30	2.55	2.16
Total circulating protein (CP <sub>t</sub> = Gm/PV <sub>t</sub> )	0.42	0.64	0.29	0.55	0.64	0.25	0.19
Unit circulating protein (CP <sub>u</sub> = Gm/PV <sub>u</sub> )	0.21	0.29	0.15	0.20	0.23	0.14	0.11
Total blood volume (BV <sub>t</sub> = cc)	10.72	16.40	9.19	13.40	15.50	6.74	6.37
Unit blood volume (BV <sub>u</sub> = cc/100 cm <sup>2</sup> SA)	5.37	7.29	4.79	4.95	5.85	3.70	3.71
Unit circulating hemoglobin (CHb <sub>u</sub> = Gm/BV <sub>u</sub> )	0.67	0.95	0.63	0.63	0.84	0.39	0.50

I infected C control

\*In general each animal was subjected to only one plasma volume study, hence the data of

†The number of animals contributing to each mean is small; the standard deviation as pre

PV<sub>t</sub> ± 0.67-1.87 PV<sub>u</sub> ± 0.35-0.46 CP<sub>u</sub> ± 0.02-0.03 BV<sub>t</sub> ± 1.07-2.48 BV<sub>u</sub> ± 0.51-0.57 CHb<sub>u</sub>

## OF BLOOD CELLS AND PLASMA PROTEIN

## INFECTION AND DIET

DAYS	14 DAYS				21 DAYS				28 DAYS				42 DAYS			
2%	18%		2%		18%		2%		18%		2%		18%		2%	
I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C
5 0	5 2	5 2	5 2	5 2	5 2	5 2	5 2	5 2	9 2	5 4	10 5	8 5	10 5	8 5	10 5	8 5
110 113	135 129	129 138	135 129	129 138	132 133	127 142	145 152	138 127	145 152	138 127	154 155	126 136	154 155	126 136	154 155	126 136
83 120	195 142	103 120	195 142	103 120	183 379	147 195	159 234	60 49	159 234	60 49	148 252	74 118	148 252	74 118	148 252	74 118
11 10	20 7	16 4	20 7	16 4	17 20	20 23	25 10	19 17	25 10	19 17	18 11	11 12	18 11	11 12	18 11	11 12
89 88	19 94	83 96	19 94	83 96	81 79	78 78	73 90	79 82	73 90	79 82	81 80	87 80	81 80	87 80	81 80	87 80
09 494	730 685	596 494	730 685	596 494	748 714	563 568	710 724	651 582	710 724	651 582	761 782	508 540	761 782	508 540	761 782	508 540

peated observations frequently derived from the same animal for the duration of the experiment  
 taken as previously determined in statistically adequate series of similar rats was  $TP \pm 0.34-0.41$  Hb

indicated at top of column

## HEMOGLOBIN VARIATIONS WITH DIET AND INFECTION\*

DURATION OF INFECTION											
14 DAYS				28 DAYS				42 DAYS			
18%	2%	18%	2%	18%	2%	18%	2%	18%	2%	18%	2%
I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C	I C
0 3	240 4	183 0	176 4	179 7	315 4	205 7	158 8	299 0	306 3	172 4	143 1
41	0 80	0 88	4 94	7 14	7 24	6 01	5 28	1 28	1 10	0 15	0 23
0 40	4 19	3 08	3 71	5 18	8 74	4 86	3 83	4 82	7 42	3 69	0 28
0 11	1 10	1 02	2 11	1 84	2 06	2 36	2 43	1 62	2 40	2 12	1 00
0 41	0 29	0 21	0 18	0 37	0 67	0 29	0 20	0 38	0 00	0 19	0 14
0 16	0 12	0 12	0 10	0 18	0 22	0 14	0 13	0 12	0 19	0 11	0 11
9 00	6 81	0 9	6 20	9 07	15 90	8 19	6 13	9 00	1 7	0 80	0 40
3 49	2 07	3 16	3 50	3 26	0 00	3 08	3 86	0 1	4 40	3 41	3 10
0 41	0 7	0 41	0 49	0 40	0 76	0 55	0 49	0 46	0 69	0 43	0 43

this table represent determinations on approximately fifty rats  
 usually determined in statistically adequate groups of similar rats was as follow  $TP \pm 0.34-0.41$   
 0.09-0.10

well-nourished and deficient animals were apparent only shortly after infection was initiated and again forty-two days later as the experiment was concluded. The immediate postinfection period was accompanied by higher hemoglobin concentrations in the infected and control protein-deficient animals (Table I). Hemoconcentration attending this phase of acute protein deficiency would seem to account for the high levels noted and serve to mask a suggestive depletion of unit circulating hemoglobin in the protein deficient groups<sup>28-29</sup> (Table II, Fig 2). However, a consistent relationship between hemoglobin concentration and unit circulating hemoglobin was not demonstrable during this early phase of rapid adjustment to infection and diet. At twenty-eight and forty-two days after infection, both hemoglobin concentrations and unit circulating hemoglobin levels appeared to be diminished in the protein-deficient animals, without significant difference being imposed by infection. This finding would seem compatible with the observed anemia associated with long-continued low casein diets<sup>28-31</sup> and is possibly related to impaired globin formation. The decreased unit circulating hemoglobin of the well-nourished infected animals was masked by the decreased plasma volume and resultant apparent hemoglobin concentration. With the exception of a probable technical error on the fourteenth postinfection day, the circulating hemoglobin of the uninfected, well-nourished rats was somewhat greater than that noted in the other groups. The relative anemia of the well-nourished infected animals may represent an anemia of infection, presumably related to hypoferrinemia<sup>32</sup>.

*Total Leucocyte and Differential Counts* Reported values for total leucocyte and differential counts in rats approximate 8 to 15,000 white blood cells per cubic millimeter, of which 4 to 25 per cent are polymorphonuclear granulocytes, the remainder, largely lymphocytes<sup>27</sup>. Infected and control animals on the 2 per cent protein diet manifested an absolute leucopenia after approximately three weeks of dietary deficiency. Leucopenia<sup>13</sup> and granulocytopenia<sup>33</sup> have been observed to attend protracted dietary protein restriction. Well-nourished uninfected rats appeared to have the highest total white blood cell counts after the second post-infection week. No other striking differences in total white blood cell counts of the various experimental groups were evident. Relative lymphopenia was observed in both groups of infected animals forty-eight hours after infection was initiated. The 18 per cent control value inadvertently represented a single animal observation. With this possible exception, the immediate post-infection lymphopenia would seem to be in accord with the alarm reaction phase of the general adaptation syndrome of Selye<sup>34, 35</sup> and may have represented a period of rapid lymphocyte dissolution with attendant elaboration of antibody globulin<sup>36-37</sup> in both groups of infected rats, although no antibody titers were demonstrable at that time.

*Hematopoiesis* Peripheral blood leucocytes and hemoglobin variations were associated with suggestive hematopoietic changes in the femoral bone marrow. The distribution of cellular elements in the femoral marrow of the twenty-seven well-nourished and twenty-five deficient rats examined was similar to that reported in twenty-four Wistar strain<sup>26</sup> and twelve Rockland strain rats<sup>38</sup>. Protracted protein deficiency was associated with a tendency toward a relative

TABLE III AVERAGE VARIATIONS IN FEMORAL BONE MARROW CYTOLOGY WITH INFECTION AND DIET

GROUP	RANGE AND AVERAGE VALUES (%) PER 500 CELLS COUNTED*											MYELOID	ERYTHROID	M E	
	NUMBER OF ANIMALS	IMMATURE MYELOID FORMS	MATURE GRANULOCYTES	ERYTHROBLASTS	NORMOBLASTS	LYMPHO CYTES†	MISCELLANEOUS								
28 days postinfection															
18% Infected	9	22.8-46.2 30.2	15.6-31.6 20.2	2.0-16.8 8.7	27.0-51.0 34.8	0.2-7.4 3.7	0.8-6.0 1.8	50.4	43.5					1.16	
18% Control	2	29.7-39.1 35.4	9.7-23.8 16.8	6.5-9.7 8.1	27.2-40.0 33.6	2.7-7.3 5.0	0.8-1.4 1.1	42.2	41.7					1.26	
2% Infected	4	3.3-42.3 39.2	18.1-31.0 21.9	2.0-10.6 5.2	21.1-40.0 29.1	1.5-7.7 3.6	1.0-2.0 1.4	61.1	34.3					1.78	
2% Control	4	27.9-60.7 43.1	3.0-36.4 19.2	3.6-14.1 9.4	22.8-31.2 26.9	0.8-2.8 1.6	1.0-5.0 2.1	61.3	34.9					1.76	
42 days postinfection															
18% Infected	9	10.8-30.8 22.9	18.4-40.4 26.9	1.2-14.4 4.0	33.2-53.3 42.0	0.6 0.6	2.6-7.0 3.9	49.8	46.0					1.08	
18% Control	7	20.0-42.6 28.5	18.4-30.8 26.0	2.4-18.2 7.3	16.8-44.2 34.3	0.6-2.4 1.4	1.8-7.0 3.1	54.5	41.8					1.30	
2% Infected	9	15.4-44.4 29.7	17.8-34.4 26.7	2.8-27.0 9.8	18.2-46.2 30.1	0.2-4.7 1.5	1.0-4.2 0	50.9	39.9					1.40	
2% Control	8	18.0-47.8 32.2	18.0-32.4 26.0	1.6-8.4 3.0	18.4-46.2 32.6	0.4-4 1.6	0.8-7.6 2.7	58.2	37.0					1.55	

Cytologic differentiation in general followed that of Stasnev and Higgins. We are indebted to Dr. A. J. and to Miss Doris Wilson for assistance with the counts. With the exception of lymphocyte counts consistent definition was achieved. Immature myeloid forms include myeloblasts, promyelocytes, metamyelocytes, and metarubricytes. Erythroid forms include erythroblasts, normoblasts, and nucleated red cells. The majority of the low lymphocyte counts in the forty two day postinfection groups were recorded by one observer.

increase of immature myeloid forms without apparent increase in mature granulocytes, either in the marrow or peripheral blood. This may have been a compensatory response to peripheral leucopenia, or might suggest a granulocyte maturation inadequacy or arrest. Depletion or inhibition of some factor necessary for production of the mature granulocyte may have been responsible. The phenomenon appeared to be associated with dietary protein deficiency irrespective of infectious status. Fewer maturing erythroid forms, particularly normoblasts, were noted in the deficient rats. The diminished erythropoietic activity of the protein deficient rat may have resulted from inadequate globin formation and may have been partially responsible for the observed depletion of unit circulating hemoglobin. At the level of hemoglobin synthesis attained, hypoferrremia associated with infection<sup>32</sup> appeared to be a less important limiting factor. The tendency of infected well-nourished animals toward a larger proportion of erythroid forms than the uninfected controls might reflect a compensatory response to relative depletion of unit circulating hemoglobin. Presumably adequate globin synthesis occurred on an 18 per cent casein diet. Accordingly, at the level of hemoglobin synthesis in the well-nourished infected rat, hypoferrremia may have been a limiting factor and partially responsible for the observed depletion of unit circulating hemoglobin.

Hematopoiesis, as reflected in the femoral marrow myeloid-erythroid differences, appeared to be more influenced by diet than by infection. The immune response observed at forty-two days postinfection did not appear to be associated with distinct variations in the femoral bone marrow picture in any of the groups.

*Plasma Proteins* Total plasma protein concentrations in rats of this age, sex, and strain vary from  $6.60 \pm 0.1$  to  $8.07 \pm 0.07$ <sup>23, 39</sup>. In this particular experiment, the total plasma protein concentration appeared to reflect the level of protein nutrition of the animal. In general, however, the measurement of concentration provides an inconsistent indication of total circulating proteins owing to plasma volume variations<sup>40, 41</sup>. Protein deficiency per se is attended by significant decreases in unit circulating plasma proteins<sup>28, 29, 42, 44</sup> (Fig. 2, Table I). Infection per se did not appear to induce significant change in total plasma protein concentrations. However, the unit circulating proteins of the infected rats on the 18 per cent protein diet were somewhat reduced after twenty-eight to forty-two days, in comparison with the appropriate controls, but exceeded those of the protein-deficient animals. Both groups of deficient animals had similar unit circulating protein levels.

Infection per se appeared to be associated with minimal change in plasma protein concentration, unit circulating protein, and electrophoretic plasma protein components. The electrophoretic distribution of plasma components, like the other protein measurements, varied with the dietary status. Fairly adequate resolution of the major plasma protein components (albumin, alpha, beta plus fibrinogen, and gamma globulins) was possible with a diethyl barbiturate buffer at pH 8.6, under the conditions of electrophoresis. More detailed differentiations of alpha-1 and alpha-2 globulins and of alpha-2 and beta globulins were less satisfactory. However, assuming six components, Gauss probability curves were con-

TABLE IV ELECTROPHORETIC MOBILITIES OF WELL NOURISHED AND PROTEIN DEFICIENT RATS

	'f'	ALBUMIN	$\alpha$	$\alpha_2$	$\beta_1$	$\beta_2 + \Phi$	$\gamma$
18% Casein diet							
Range	7680	5763	4753	4146	3438	2526	1415
Mean	79	60	50	43	36	26	14
2% Casein diet							
Range	7177	5560	4350	3236	3742	2226	0915
Mean	73	59	48	35	41	25	13

Mobilities calculated from descending boundary patterns only according to the formula  $\Delta h \times \frac{1}{T \text{ sec}} \left( \frac{0.5}{\text{Milliamperes}} \right)$  hence all values represent  $M \times 10^5$ . The lower values in the range of per cent diet plasma mobilities are contributed entirely by the 2 per cent control group after twenty eight days of diet deficiency. This analysis was run some months after those of the seven other groups. All means are weighted by relative numbers of animals per group contributing to the value.

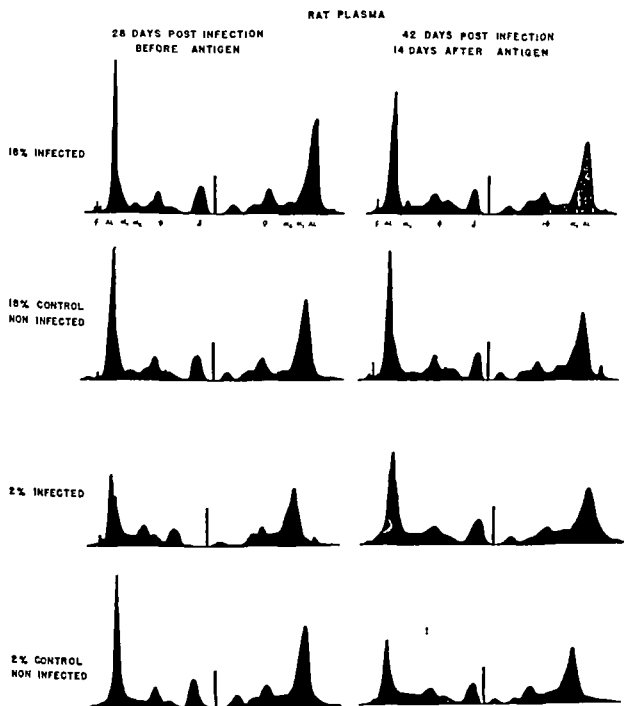


Fig 3—Schlieren patterns of the electrophoretic analyses of rat plasma pools derived from infected and noninfected rats on 18 per cent and 2 per cent protein ration. Both ascending and descending boundary patterns are shown twenty-eight days after infection (prior to antigenic stimulation) and fourteen days later following the injection of *S typhimurium* O antigen. All determinations were made using a sodium diethyl barbiturate buffer at pH 8.6 and 0.10 ionic strength. The 2 per cent diet infected preantigen pattern and the 2 per cent diet control postantigen pattern represent protein concentrations of 1 and 1.4 per cent respectively. The remainder of the patterns represent analyses at  $2.0 \pm 0.1$  per cent protein concentrations.



TABLE V ELECTROPHORETIC VARIATIONS IN

GROUP	NUMBER OF ANIMALS	PLASMA PROTEIN CONCENTRATION (GM / 100 CC)	PER CENT PLASMA COMPOSITION *							
			“f”	ALBU MIN	$\alpha_1$	$\alpha$	$\beta_1$	$\beta + \Phi$	$\gamma$	TOTAL
28 days postinfection (before second antigen)										
18% control	2	6.46	3.0	54.5	10.9	5.6	3.6	15.2	7.2	99.9
18% infected	9	6.78	2.9	51.0	11.6	7.7	4.6	15.3	7.4	99.5
2% control†	4	5.18	5.4	60.8	5.9	4.3	3.3	14.8	5.5	100.0
2% infected	4	6.28	6.9	43.2	12.0	6.6	5.7	18.0	7.7	100.1
42 days postinfection (after second antigen)										
18% control	5	7.12	4.2	57.0	6.7	7.2	3.0	15.2	6.9	100.2
18% infected	10	7.36	3.4	55.6	5.9	4.5	3.5	16.1	10.7	99.7
2% control	5	5.68	3.3	49.0	11.5	6.1	5.8	18.4	6.0	100.1
2% infected	8	5.13	8.5	48.7	9.8	5.8	5.3	16.7	5.3	100.1

\*Somewhat arbitrary values derived from relative areal resolution and representing average per cent.

†Derived from the product of the plasma protein concentration of the pool and the per cent.

‡Represents the product of the average unit circulating proteins (Table II) derived from the of the resolved schlieren pattern.

§Two per cent control calculations were derived from electrophoretic runs carried out several

sistently and reasonably, if somewhat arbitrarily, drawn. Separation of beta-1 and beta-2 globulins was not entirely satisfactory in these analyses, although distinct demarcations were apparent in some instances. Beta-2 globulin and fibrinogen were not separated under the conditions of electrophoresis and, accordingly, were considered together. Gamma globulin was quite distinct in all patterns and was well separated from the boundaries. A so-called "f" component with mobility greater than that of albumin has been noted in rat plasma<sup>45</sup> and consistently appeared in our patterns. Its significance is not known. The mobilities of the various components (Table IV), calculated from the descending patterns, are similar to those reported for other rats<sup>45, 46</sup> and roughly approximate the mobilities of human plasma components.

The plasma composition is indicated in Table V. Typical patterns are illustrated in Fig. 3. Since all electrophoretic data were derived from pooled samples, the range and standard deviation of individual rat variation were not evident. The composition of rat plasma appears to vary with the strain used.<sup>46</sup> Previous reports have been largely concerned with the Long-Evans<sup>46, 47</sup> or Sprague-Dawley strains.<sup>48</sup> In the Sherman strain rat used in this study, the plasma composition was similar to that observed in man,<sup>48, 50</sup> gamma globulin values were slightly lower.

Concentrations of the various plasma components were calculated from the proportionate composition of schlieren patterns and the total plasma protein concentrations of the respective pools. Since unit circulating protein data appear to be more reliable than simple concentration measurement as an indicator of plasma protein variation, the total circulating amount of each plasma component in the vascular compartment was also calculated and adjusted to unit value. Such arbitrary calculation was dependent on resolution of electrophoretic

## PLASMA PROTEINS WITH INFECTION AND DIET

CONCENTRATION OF PRINCIPAL PLASMA PROTEIN COMPONENTS (GM/100 CC)†							UNIT CIRCULATING PLASMA PROTEIN COMPOSITION (GM/UNIT PLASMA VOLUME)‡								
"f"	ALBU MIN	$\alpha$	$\alpha_2$	$\beta$	$\beta + \Phi$	$\gamma$	"f"	ALBU MIN	$\alpha_1$	$\alpha$	$\beta_1$	$\beta_2 + \Phi$	$\gamma$	TOTAL	
0.19	3.53	0.70	0.36	0.23	0.98	0.47	0.006	0.120	0.024	0.012	0.008	0.033	0.016	0.22	
0.20	3.45	0.79	0.52	0.31	1.04	0.50	0.005	0.092	0.021	0.014	0.008	0.029	0.013	0.18	
0.28	3.14	0.31	0.22	0.17	1.10	0.28	0.007	0.079	0.008	0.006	0.004	0.019	0.007	0.13	
0.43	2.71	0.75	0.41	0.36	1.13	0.48	0.010	0.061	0.017	0.009	0.008	0.025	0.011	0.14	
0.30	4.06	0.48	0.51	0.21	1.08	0.49	0.008	0.108	0.013	0.014	0.006	0.029	0.013	0.19	
0.25	4.10	0.43	0.33	0.26	1.18	0.51	0.004	0.067	0.007	0.005	0.004	0.019	0.013	0.12	
0.19	2.78	0.65	0.35	0.38	1.02	0.34	0.004	0.054	0.013	0.006	0.006	0.022	0.007	0.11	
0.44	2.51	0.51	0.30	0.27	0.86	0.27	0.009	0.054	0.011	0.006	0.006	0.018	0.006	0.11	

cent composition of ascending and descending boundary patterns

areal composition of the resolved schlieren pattern

plasma protein concentration plasma volume and surface area and the per cent areal composition

months after the other seven determinations

components, plasma protein concentrations, and mean unit plasma volumes (Table V). Accordingly, these data are more relative than absolute, owing to limitations in the various techniques employed. The results remain proportional, however, since the error was systematic.

Severe protein deficiency protracted for four to six weeks resulted in a considerable decrease in total plasma protein concentration and in unit circulating protein. This diminution in protein usually has been considered the result of albumin depletion.<sup>43-44</sup> In the electrophoretic patterns as noted by Bieler and co workers,<sup>51</sup> nutritional hypoproteinemia did not appear to be characterized by a marked decrease in the proportionate area representing albumin. However, with calculation of the relative concentration (grams per 100 cc) of the various electrophoretic plasma protein components it was evident that protein deficiency was characterized by a reduction in electrophoretic albumin as had been noted in dogs.<sup>52-54</sup> Albumin depletion appeared more marked when interpreted in relation to the associated diminished plasma volume (unit circulating plasma protein composition).<sup>\*</sup> The proportion of the pattern and the concentration of alpha and beta globulins appeared slightly increased in the hypoproteinemic rats. This observation is in general accord with that of Chow.<sup>54</sup> When adjusted to unit plasma volume, no distinct difference between circulating alpha and beta globulins in the well nourished or deficient animals was noted. The most marked electrophoretic change induced by protein deficiency was the reduction in both concentration and unit circulating amounts of gamma globulin. This relative depletion was most evident, as might be expected after six weeks of severe protein deficiency. No significant change in gamma globulin in

\* The relatively high albumin of the noninfected rats on the twenty eighth day of the per cent protein diet remains unexplained. This run having the slowest mobilities of the series was made several months after the other determinations.

response to infection per se was noted in the electrophoretic patterns of either the well-nourished or deficient groups

*Bacteriologic and Immunologic Responses*—Infection following inoculation with *S. typhimurium* was corroborated by blood culture. The time incidence and duration as observed in the 114 animals comprising this and the preliminary experiments were somewhat variable in individual animals inoculated with the same initial dose of organisms. In some instances bacteremia was present at twenty-four hours, in others, it did not appear until forty-eight hours had elapsed. It was demonstrable for three to ten days. In this study, blood culture of infected rats on the 18 per cent protein ration revealed demonstrable bacteremia in all those cultured at forty-eight hours. One of five protein-deficient infected animals did not have a positive blood culture at forty-eight hours. At four days one of five well-nourished and three of five deficient rats had positive blood cultures. The presence of bacteremia at twenty-eight days postinfection in a single protein-deficient animal remains unexplained. (See Fig. 2.) No cultures of the noninfected control rats were positive (Table VI).

TABLE VI BACTERIOLOGIC AND IMMUNOLOGIC MANIFESTATIONS

DIET PROTEIN LEVEL	POSITIVE BLOOD CULTURE				AVERAGE AGGLUTININ TITER*							
	18%		2%		19%				2%			
	IN FECTED	CON TROL	IN FECTED	CON TROL	INFECTED		CONTROL		INFECTED		CONTROL	
INFECTIOUS STATUS	H†	O‡	H†	O‡	H†	O‡	H†	O‡	H†	O‡	H†	O‡
Number of animals	5	2	5	2	5	5	2	2	5	5	2	2
Preinfection control period	—	0	—	0	—	—	0	0	—	—	0	0
		(3)§		(3)			(6)	(6)			(6)	(6)
Postinfection												
48 hours	5	0	4	0	0	0	0	0	0	0	—	—
5 days	1	0	3	0	66	90	0	0	133	80	0	0
					(3)	(4)	(1)		(3)			
10 days	0	0	0	0	120	90	0	0	180	152	0	0
					(4)				(4)			
14 days	0	0	0	0	40	88	0	0	88	64	—	0
							(1)					
21 days	0	0	0	0	60	4	0	0	208	144	0	0
28 days	0	0	1	0	276	44	0	0	232	44	0	0
	(9)				(9)	(9)					(4)	(4)
42 days	0	0	0	0	400	200	4	4	356	328	0	4
	(10)	(6)	(9)	(5)	(10)	(10)	(5)	(5)			(5)	(5)

\*On twofold dilution basis: first tube 1:20 second 1:40 etc. Titers are reported as the reciprocal of the average titer of the group.

†H indicates flagellar antigen agglutination.

‡O indicates somatic antigen agglutination.

§Number in parentheses indicates number of animals represented in determination when that figure differs from number indicated at top of column.

||Forty-second day titers represent determinations made fourteen days after injection of O antigen subcutaneously in all animals on the twenty-eighth postinfection day.

No very marked difference in the incidence of bacteremia was noted in the respective dietary groups. Two spontaneous deaths occurred during the postinfection phase, both were in protein-deficient rats. Only one was an infected animal and it died on the ninth postinfection and fifteenth diet deficient day. The other, noninfected, died on the twenty-seventh day of dietary deficiency. In preliminary experiments, an approximate 50 per cent mortality was observed in both diet groups. The majority of those spontaneous deaths occurred before the third postinfection day in the deficient rats. In the well-nourished rats,

sporadic deaths were noted over a ten day period. The low mortality of this study would appear to be the result of the relatively small inoculum of viable cells. The host<sup>14</sup> and pathogen<sup>55</sup> variation from experiment to experiment cannot be adequately evaluated. The route of infection appears to be of considerable importance in relation to the subsequent clinical course. In per os infections with *S. typhimurium* demonstrable bacteremia rarely occurs. Extension of infection is largely lymphatic. Diarrhea may characterize the clinical course. With intraperitoneal inoculation dissemination of organisms is apparently largely hematogenous and diarrhea is not a constant finding.<sup>56</sup>

The average agglutinin titer to somatic and flagellar antigens is tabulated according to the reciprocal of the average titer of the group (Table VI). The trend of antibody response to infection is depicted in Fig. 2. It is evident that both O and H agglutinin titers were demonstrable at five days postinfection in both deficient and well nourished rats. The highest recorded titer in both groups at that time was 1:160. Titers remained relatively constant averaging approximately 1:160 for twenty one days after infection. H agglutinin titers in both deficient and well nourished animals were somewhat higher than O agglutinins. The difference was more marked at twenty eight days, flagellar agglutinins in some instances attaining titers of 1:640 (seven tubes) in both diet groups. These data suggest that severe protracted protein deficiency per se did not impair circulating humoral antibody response to the virulent infecting pathogen used in these studies. Lack of significant difference in quantitative circulating gamma globulins in the two diet groups appears consistent with the observed antibody responses at twenty eight days after infection.

On the twenty eighth postinfection day all animals were inoculated subcutaneously with O antigen in order to test the immune and anamnestic responses. The average response of the deficient infected animals as measured by O and H agglutinin titers was slightly greater than that of the well nourished animals. The highest titers were 1:640 and 1:1280. The increase in O agglutinin titers, corresponding to an immune response was more marked than the associated rise in H titers. The apparent immune and anamnestic agglutinin responses did not appear to correlate with the changes in quantitative circulating gamma globulin in either group. Unit circulating gamma globulin of the 18 per cent protein rats remained essentially unchanged, that of the 2 per cent protein rats was distinctly diminished, despite increased agglutinin titers in both groups. No significant differences in circulating gamma globulin were noted between infected and control animals of respective diet groups although the control groups had negligible or no agglutinin titers at either twenty eight or forty two days. These data might be consistent with observations that the O antibody to another bacterial species, *Escherichia typhosa*, is not contained in the gamma globulin fraction<sup>5</sup> or that all gamma globulin is not antibody.<sup>5</sup> In this regard, it is interesting that the somatic antibody is probably more closely related to protection against infection than is the flagellar antibody.<sup>5</sup> The secondary rise in O titer following inoculation of type specific O antigen in the hypoproteinemic rats would seem to indicate that dietary protein deficiency does not seriously impair the mechanism for protective antibody fabrication in

the rat. This finding is at variance with that reported by Cannon and co-workers<sup>14, 15</sup> and Berry and associates<sup>12</sup> in rats, and by Klebs<sup>60</sup> in one patient. It is in accord with the conclusions reached by Bielei and co-workers<sup>51</sup> with several human subjects with hypoproteinemia.

#### COMMENT

Protein deficiency causing inadequate response to infection would appear to be an oversimplification of a many-faceted phenomenon. An appropriate experimental procedure involving the single variable of protein deficiency has not yet been reported. The genetic status of the host<sup>16</sup> and pathogen,<sup>56</sup> host factors initiating antibacterial immunity,<sup>59</sup> the source of protein,<sup>8</sup> protein deficiency conditioning other nutritional inadequacies as for example that of macin,<sup>61</sup> and numerous other fundamental variables have not been simultaneously controlled. Endocrine, protein, metabolic interrelationships<sup>56</sup> and coincident nitrogen balance, liver protein, and protein synthesis studies would be helpful in interpreting the place of protein in infection response.<sup>62</sup>

This study represents an attempt to correlate a few related phenomena. Data derived from this and the preliminary studies manifest some degree of reproducible consistency, however, conditions attending the observations necessitate some degree of statistical inadequacy. The most satisfactory absolute measures of experimental infection with a virulent pathogen are noninfection or death—complete resistance or complete susceptibility—as specific unequivocal end points. However, physiologic variations associated with such an infection must be measured in the mid-ground between complete resistance and susceptibility, and hence are dependent upon continuity and interrupted by death. The data of this study, therefore, are not strictly comparable to those of Schneider and Webster,<sup>16</sup> Sako,<sup>11</sup> or Watson<sup>8</sup>; their data were concerned with unequivocal susceptibility or resistance, this study with neither. Nor is it comparable with the data of Cannon and co-workers<sup>14, 15</sup> or Berry and associates,<sup>12</sup> since in their studies infection was not initiated.

The response to specific *Salmonella* infection of the protein deficient rat, as demonstrated by Zilva,<sup>63</sup> Lassen,<sup>56</sup> and Guggenheim and Buechler<sup>13</sup> and somewhat extended by this report, is apparently not markedly different from that of the well-nourished control. Physiologic variations in circulating protein observed in the *Salmonella*-infected protein-deficient rats of this experiment appear to be adequately explained by the protein deficiency per se and were comparatively uninfluenced by infection. Hematologic and hematopoietic responses, on the other hand, appear to be influenced to some extent by both diet and infection. Bacteriologic and immunologic responses, in contrast to those of the plasma proteins, did not appear to be significantly altered by protein deficiency. It is perhaps irrelevant to indicate that the crude measures of bacteremia and survival are not the only manifestations of bacteriologic response, and antibody titer does not wholly define antibacterial immunity. The multiplicity of subtle changes involved in both protein deficiency and infection precludes such oversimplification of a possible interrelationship. *Salmonella* in-

fection, for example, is presumably an intracellular infection involving mononuclear cells of lymphoid follicles. Protein deficiency of the type initiated might not sufficiently damage these cells which presumably mediate both the course of infection and antibody response.

#### SUMMARY

Rigid conclusions cannot be drawn from a biologic study involving isolated changing responses, rather than a specific unequivocal end point. The data presented are consistent with those observed in preliminary experiments. The data on growing Sherman strain rats subjected to infection with virulent *S. typhimurium* and to either severe protracted protein deficiency at nitrogen intakes varying from 19 to 39 mg nitrogen per 100 Gm rat per 24 hours, or adequate nitrogen intakes approximating 155 to 368 mg nitrogen per 100 Gm rat per 24 hours, may be summarized as follows:

1 Quantitative circulating plasma proteins particularly gamma globulins in both well nourished and protein deficient animals appeared to be relatively uninfluenced by infection per se despite observed changes attending protein deficiency. With protracted protein deficiency, decreased unit circulating plasma proteins, apparently representing a depletion of albumin and gamma globulin components, were observed independent of infection.

2 In both well nourished and protein deficient rats, hematologic responses as evidenced by differential bone marrow counts, total and differential leucocyte counts, and circulating hemoglobin values appeared to be altered somewhat by both infection and diet. Decreased unit circulating hemoglobin was noted in both well nourished and deficient infected rats. The diminution was somewhat more marked in the protein deficient animals. Total leucocyte counts of the deficient groups were somewhat lower than those of the well nourished. The initial postinfection relative lymphopenia manifested by both infected groups was similar.

3 Hematopoiesis, as reflected in the femoral marrow myeloid erythroid differences, appeared to be influenced more by diet than by infection. Protein deficiency was associated with a tendency toward a relative increase of immature myeloid forms and decreased numbers of maturing erythroid elements. Well nourished infected animals appeared to have a relatively greater number of maturing erythroid forms than the controls.

4 Bacteriologic and immune responses resulting from infection and secondary antigenic stimulation were essentially unaltered by severe dietary protein deficiency. No significant difference in the incidence of bacteremia was noted between infected rats on the 2 per cent or the 18 per cent casein diets. Despite relative quantitative depletion of circulating gamma globulin, protein deficient rats attained antibody titers equivalent to those of the well nourished animals in response to infection. Following specific secondary antigenic stimulation the rise in humoral antibody titer was more marked in the deficient than in the well nourished rat. Despite rise in titer no significant change in circulating gamma globulin was observed in either group.

5 In general, pertaining to the moieties tested, rats undergoing severe protein deprivation appeared to respond similarly to and as adequately as well nourished controls to infection with *S typhimurium*

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## HIGH VITAMIN A INTAKE AND BLOOD LEVELS OF CHOLESTEROL, PHOSPHOLIPIDS, CAROTENE, AND VITAMINS C, A, AND E

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THE literature contains several reports relating vitamin A to the blood and tissue lipids. Josephs<sup>1, 4</sup> has reviewed most of the pertinent articles and from them and his own work concluded that the hypolipemia seen in a vitamin A deficient animal is due to a specific pathologic effect of an actual internal deficiency of vitamin A. It was found that the abrupt administration of large amounts of this vitamin caused a marked rise in total serum lipids of both normal and vitamin A deficient rats. Josephs<sup>3</sup> suggested a simple fat solubility relationship between the vitamin and other lipids to account for this effect of vitamin A. Green,<sup>5, 6</sup> in studies on serum esterase and fat absorption, and Monaghan and Schmitt, who investigated the effect of vitamin A upon unsaturated fatty acids, were inclined to relate vitamin A action to metabolic processes of essential cellular structures involving phospholipid complexes.

A number of workers have not been able to demonstrate the hypolipemia of vitamin A deficiency and the hyperlipemia of high vitamin A intake. Smith<sup>8</sup> in fact, found an increase in blood fatty acids and cholesterol in deficient rats and Sure Kik and Church<sup>9</sup> found no change in blood fatty acids, cholesterol and phospholipids in vitamin A deficient albino rats. Rall and Waterhouse<sup>10</sup> found an increase of blood cholesterol in deficient dogs.

If the hyperlipemia observed by Josephs in a child<sup>4</sup> and the hypercholesterolemia induced by vitamin A in the patients studied by Lasch<sup>11</sup> and Juszat<sup>1</sup> are a normal response in man to large doses of the vitamin then a study of this response may help to uncover the role of this vitamin in human biochemical processes. In addition, if this blood lipid response is a general phenomenon induced by ingestion of vitamin A the extent of these changes needs to be determined because of the association of hyperlipemia and hypercholesterolemia with important pathologic processes.

That vitamin A may be interrelated with a number of other vitamins is clearly pointed out in the review of this subject by Moore.<sup>12</sup> In the present study an attempt has been made to investigate not only the relation of vitamin A intake to blood lipid levels but also to the blood levels of several of the other vitamins interrelated with vitamin A.

*General Considerations*—We have had the opportunity of studying the effects of daily supplementation with 100,000 I.U. of vitamin A on a group of patients in the Western State Hospital†. Some of the effects of this supple-

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†The patients and facilities of the hospital at Steilacoom, Wash., were kindly put at our disposal by Dr. W. N. Keller for a long term study of the effects of vitamin A supplementation. Without the assistance of Dr. William J. Dublin and Dr. Bernice M. Hazen this work would not have been possible.

mentation on the skin of these patients will be reported by Dublin and Hazen<sup>14</sup> The present paper is concerned with a comparative study of the blood vitamins A, E, and C, cholesterol, and phospholipid levels in a group of patients supplemented with 100,000 units of vitamin A daily and in an unsupplemented group of patients We have been unable to find in the literature any reports of work in which supplementation with large doses of vitamin A was continued over a long period of time and in which blood assays for these vitamins and for lipids were performed on a sufficient number of individuals to warrant a statistical treatment of the results

The number of patients in each group at the beginning of the study was thirty-six The patients in these groups were in similar mental and physical condition as indicated by examination and distribution curves for age, height and weight

The supplemented group received at all times the same institutional diet as the control group but, in addition, one capsule\* daily containing 100,000 I U of vitamin A Determinations† of plasma carotene, vitamin A, vitamin E, free and total cholesterol, and phospholipid as well as whole blood vitamin C were done on five occasions—once before supplementation was started and after intervals of approximately eighteen, twenty-four, and thirty-six months of supplementation Additional determinations were made at forty-two months, six months after supplementation was discontinued The analyses of vitamins and lipids made at the beginning of the experiment as well as the lipid analyses at the eighteen-month period have been rejected because of unsatisfactory analytical methods

Since the blood samples were taken from the patients at Steilacoom, Wash, and a period of four to six hours necessarily elapsed before analytical work could be started in the laboratory at Astoria, Ore, the preparation and storage of blood samples will be described briefly Fifteen to twenty milliliter samples of blood were collected from each patient of a group of fifteen to twenty five patients approximately sixteen to eighteen hours after the last meal and sixteen to twenty four hours after the last dose of vitamin A Coagulation was prevented by mixing with oxalate in 30 ml centrifuge tubes dried as described by Van Bruggen<sup>15</sup> Two milliliter samples of whole blood were immediately added to 6 ml of 6 per cent trichloroacetic acid containing norit After thorough mixing, the samples were stored on ice for transport The plasma from the remaining blood was separated and 1 ml was added to 24 ml of alcohol ether (3:1) in a 50 ml glass stoppered cylinder Immediately upon arrival at Astoria the trichloroacetic acid samples were filtered and the filtrates refrigerated for twenty four to forty eight hours until the vitamin C analyses could be made The alcohol ether extracts were filtered upon arrival at Astoria and analysis for cholesterol and phospholipid was begun immediately The remaining plasma was kept on ice until the analytical work for carotene and vitamins A and E had been completed These analyses were usually finished within forty eight hours

Since only twenty to twenty five blood samples could be assayed at one time, a complete assay of the two groups required three to four weeks at intervals of 0, 18, 24, 36,

\*The vitamin A capsule known as Oleum A is prepared by Bioproducts Oregon Ltd Warrenton Oregon Analysis shows this product to contain the following constituents all analyses being reported as per capsule

Weight of contents 0.430 Gm free cholesterol 28 mg total cholesterol 84 mg phospholipid 0.65 mg vitamin E 0.67 mg vitamin A 100,000 I U (equivalent to 33 mg of pure vitamin A)

†We gratefully acknowledge the assistance of Distillation Products Inc Rochester N Y in the establishment of carotene and vitamin A and E methods We are especially grateful to Dr Mary L Quaife of that organization for her practical help on this problem

and 42 months, so that the time periods listed in this paper are given as approximately eighteen, twenty four, thirty six and forty two months. To insure comparable results an equal number of patients in each group was sampled at each a stay trip.

### METHODS

Petroleum ether extracts of 4 ml of plasma were prepared for the determination of carotene, vitamin A, and vitamin E. The plasma was well mixed with 4 ml of alcohol and then extracted once with 12 ml of purified low boiling petroleum ether. Extraction was done in a 50 ml glass stoppered cylinder by shaking for ten minutes in a horizontal position in a mechanical shaker at a speed of 160 strokes per minute with an excursion of three inches per stroke. Refrigeration of the cylinders for one hour insured good separation of the layers. Four milliliters of the petroleum ether extract were used for carotene and vitamin A determinations and 4 ml for vitamin E. Carotene was measured at 440 m $\mu$  and after the solvent was evaporated at 60 C under nitrogen the vitamin A was estimated by the Carr Price reaction (that is, the residue was taken up in 0.5 ml of dry chloroform, 1 drop of acetic anhydride was added and 3.5 ml of Carr Price reagent were added from an all glass apparatus that insured rapid yet accurate delivery of an anhydrous reagent). Vitamin A values were corrected for the blue color contribution of the carotene present in the sample.

Vitamin E was determined on the 4 ml aliquot of the petroleum ether extract by the method of Quarte and Harris,<sup>18</sup> modified only in that smaller volumes of extract and reagents were used. The interference of vitamin A and carotinoids was eliminated by catalytic hydrogenation as described by Quarte and Biehler.<sup>1</sup>

Standard curves for vitamin A, carotene and vitamin E were prepared and frequently checked during the progress of the assays.

Whole blood vitamin C was determined by our semimicro modification of the method of Roe and Kuether.<sup>18</sup> The final total color volume was 4 milliliters.

Free and total cholesterol were determined by the digitonin method of Sperry<sup>19</sup> with minor modifications. The use of alcoholic solutions of digitonin as suggested by Sobel and Mayer<sup>20</sup> and the washing of precipitates by solutions forcibly ejected from a syringe and needle made the use of individual stirring rods unnecessary.

The lipid phosphorus fraction was obtained by evaporation of the alcohol ether filtrate extraction with petroleum ether and precipitation with acetone and magnesium chloride.<sup>21</sup> Final phosphate determinations were made with the reagent described by Comori.<sup>2</sup> The factor of 26 was used for conversion to lipid phosphorus.

Many of the various analyses were conducted in duplicate with good agreement.

### RESULTS

Since the data presented below represent the results of some 1600 determinations, space does not permit the tabulation of individual determinations. Table I gives the values of the various assays  $\pm$  their standard deviation. Significance of differences in the supplemented and unsupplemented groups is indicated by "t" values.

It is apparent that although the supplemented patients received daily an amount of vitamin A at least ten or twenty times that contained in the diet of

<sup>18</sup> All colorimetric determinations were done with a Model 11 Coleman spectrophotometer. It is possible to use 4 ml volumes in the large cuvettes of this instrument without affecting the sensitivity if the cuvette is raised by a suitable cut in the cuvette holder and the 0.1 in. of the holder is masked to exclude the rounded portion of the cuvette and the unfilled portion of the cuvette from the path of the incident light.

<sup>19</sup> To obtain reasonably stable Carr Price end points with the Model 11 instrument it is necessary to reduce the intensity of the incident light to a minimum value as recommended by Callwell and Parri.<sup>17</sup>

<sup>21</sup> Pure tocopherol crystalline carotene (90 per cent 10 per cent) and a distilled vitamin A concentrate containing 0.1500 IU per gram were kindly supplied by Distillation Product Inc. Roche ter.

TABLE I BLOOD LEVELS OF CAROTENE, VITAMINS A, E, AND C, CHOLESTROL, AND PHOSPHOLIPID OF INSTITUTIONAL PATIENTS, ONE HALF OF WHOM RECEIVED A CAPSULE CONTAINING 100,000 UNITS OF VITAMIN A A DAY

	18 MONTHS					24 MONTHS					36 MONTHS					42 MONTHS*				
	N	M	$\pm$	$\sigma$	t	N	M	$\pm$	$\sigma$	t	N	M	$\pm$	$\sigma$	t	N	M	$\pm$	$\sigma$	t
Vitamin A (IU %)	36	149	40.9		7.95	35	145	53.4		7.1	30	135	31.0		8.0	29	123	36.7		3.2
Control	35	256	68.3			33	307	94.5			25	304	101			24	156	35.7		
Supplemented	36	216	66.5	0.86		35	213	60.8	0.43		30	193	57.4	0.95		29	213	65.5	0.43	
Control	36	203	60.3			33	207	53.7			25	208	64.2			24	206	67.5		
Supplemented	35	104	0.25	2.5		35	0.98	0.24		3.4	30	1.01	0.17		4.7	29	0.99	0.19		0.2
Control	36	120	0.26			33	1.20	0.28			25	1.27	0.22			24	1.09	0.23		
Supplemented																				
Vitamin C (mg %)						31	0.87	0.36	0.13		27	0.99	0.36	0.07		24	0.64	0.35	0.09	
Control						32	0.56	0.31			25	0.99	0.35			21	0.59	0.31		
Supplemented																				
Free Cholesterol (mg %)						34	53.4	16.9		2.0	30	53.0	10.9	3.5		29	50.8	12.3	1.1	
Control						33	60.8	15.7			25	63.8	11.6			24	54.6	14.3		
Supplemented																				
Total Cholesterol (mg %)																				
Control						34	207	44.7		2.2	30	202	39.5	3.9		29	208	42.5	1.2	
Supplemented						33	230	42.6			25	247	43.7			24	223	47.6		
Ratio F/T																				
Control						34	25.6	4.4	0.83		30	26.3	3.2	0.44		29	24.3	8.8	0.04	
Supplemented						33	26.4	4.1			25	26.0	2.5			24	24.4	9.2		
Phospholipid (mg %)																				
Control						34	220	63.4	1.37		30	218	32.3	2.4		29	210	50.7	0.6	
Supplemented						33	243	72.7			25	246	47.9			24	219	53.9		

N Number of patients assayed

M Mean of N observations  $\pm$  standard deviation which is equal to  $\sqrt{\frac{\sum (v-M)^2}{n-1}}$

t, Fisher's test of the significance of differences in practice it is equal to  $\frac{D}{E_0}$  (difference of means)  $\frac{D}{E_0}$  (standard error of difference) For numbers of samples between 25 and 30 a factor of 2 is assumed to be significant ( $P < 0.05$ )

\*At thirty six months the vitamin A given to supplemented patients was discontinued

the unsupplemented patients, the blood level of vitamin A in the supplemented group was less than three times that in the unsupplemented group. This difference, however, was maintained throughout a period of thirty six months of supplementation. In the six month period when supplementation was withdrawn, the blood level of vitamin A of the supplemented group fell to a value approximating that of the unsupplemented patients.

Plasma carotene and whole blood vitamin C seem to be entirely unaffected by the supplementation, and the minor fluctuations in these two substances seen in both groups, no doubt reflect seasonal variations in the dietary. The assays at twenty four and thirty six months were done in the fall and those at eighteen and forty two months in the spring.

The small but statistically significant increases in the vitamin E level of the blood of the supplemented patients have not been reported previously. That this increase was due to the supplementation is evidenced by the abrupt return to the control level when vitamin A was withdrawn.

The supplemented patients showed a significant increase in blood cholesterol and phospholipid. At twenty four months and thirty six months the supplemented group was 11 per cent and 22 per cent higher in total cholesterol and 10.5 per cent and 12.8 per cent higher in phospholipid than the control group. Since these increases in cholesterol occurred in both free and total cholesterol fractions, the F/T ratio remained normal. Although the increase in cholesterol was statistically significant at twenty four months the phospholipid values did not become significantly different until thirty six months. The decrease in plasma levels of both cholesterol and phospholipid of the supplemented patients after discontinuance of supplementation suggests a specific effect of the supplement upon the level of these lipid constituents.

#### DISCUSSION

Glover, Goodwin and Morton<sup>5</sup> have investigated the relation of plasma vitamin A levels to those of liver stores in rats and point out that the blood level of vitamin A is proportional to the amount of free or alcohol form present in the liver and not to the total liver stores of the vitamin.<sup>6</sup> They suggest that the free or alcohol form of the vitamin is the 'functional' form and that to supply body tissues with greater amounts of vitamin A there must be an increase in blood vitamin A alcohol. These workers found that an increase in post absorptive free vitamin A in the blood can be obtained only by massive dosing which by virtue of the alcohol ester equilibrium in the liver increases the blood vitamin A alcohol content. This fact appears to be related to the prolonged period of treatment necessary in certain skin diseases which has been reported.<sup>7, 28</sup>

A 'sparing' or 'synergistic' action of vitamin I upon vitamin A and carotene has been reported by Helman and co workers,<sup>9</sup> and Harris and associates<sup>30</sup> and this subject has been reviewed by Moore.<sup>12</sup> That vitamin A has a similar effect on vitamin E is suggested by our data. Both these substances have been shown to have a similar protective effect on unsaturated fatty acids.<sup>31</sup> It is possible that the increased blood level of vitamin I with vitamin A sup

plementation may be related to an antioxidative effect of vitamin A upon the oxidative destruction of vitamin E or to decreased utilization or to both

Under the conditions of our experiment, there appears to be a clearly significant increase in plasma cholesterol and phospholipid as a result of vitamin A supplementation. Whether these increases are a direct effect of vitamin A upon the oxidative, storage, or transport mechanisms of lipids is unknown. Chaher, Jeune, Simon, and Alacoque<sup>32</sup> and Wendt<sup>33</sup> suggested a relation of vitamin A to the thyroid gland. Recently Sadhu and Brody<sup>31</sup> reported that doses of 30,000 units of vitamin A a day to rats decreased thyroid size and basal metabolism. From this work they postulate a direct relationship between vitamin A, thyroid, and thyrotropic hormone. Such a relationship might account for a lowered tissue oxidation of metabolites and thus lead to an increase in blood levels of lipids. It must be kept in mind, however, that doses of this order of magnitude are close to the toxic level for rats and far exceed the nontoxic amounts used in our study.

Although the cholesterol and phospholipid levels of the supplemented patients were significantly higher than those of the unsupplemented patients, it should be pointed out that the mean values are within normal range. The relation, moreover, between the several lipids is normal. Peters and Van Slyke<sup>35</sup> have charted ratios for both normal and abnormal amounts of cholesterol and phospholipid. Their lipid phosphorus cholesterol ratio charted against F/T cholesterol ratios defines the limits of normal variations of these lipids. Our figures, both for supplemented and unsupplemented patients, fall within their normal area number 5. This indicates that the hyperlipemia induced by 100,000 units of vitamin A daily lies within normal limits.

The effect of supplementation for periods greater than thirty-six months cannot yet be stated with any accuracy. Preliminary treatment of data obtained from patients in the private practice of one of us (J. V. S.) who were taking 100,000 units of vitamin A daily for 5 to 10 years, indicates plasma vitamin A, vitamin E and lipid levels indistinguishable from those of patients supplemented for three years.

As far as we know, the 100,000 units of vitamin A in the capsule used in this work was the only significant added substance in the dietary intake of the patients. The cholesterol and phospholipid and oil content of the capsule was only a small increment of the daily intake of these substances and the vitamin E content was approximately 3 per cent of the estimated human daily requirement.<sup>36</sup>

#### SUMMARY

Thirty-six unsupplemented patients and thirty-six patients supplemented with 100,000 units of vitamin A daily for thirty-six months were studied. Blood assays at eighteen, twenty-four, and thirty-six months and a final assay at forty-two months, six months after supplementation was discontinued, are reported.

1. It was found that the mean plasma vitamin A level after thirty-six months of supplementation was 125 per cent higher than that of the unsupplemented patients. Six months after supplementation was discontinued, the mean levels, although still statistically higher than those of the controls, had fallen to half of their peak values.

2 No effect upon the carotene and vitamin C blood levels was observed

3 After eighteen, twenty four and thirty six months the vitamin E levels of the supplemented patients were 15.4, 22.5, and 25.7 per cent, respectively, higher than control levels

4 Both free and total plasma cholesterol levels of the supplemented patients were increased at twenty four and thirty six months to the extent of 13.8 and 20.8 per cent for free and 11.1 and 22.3 per cent for total cholesterol. F/T ratios, however, remained normal. Each of the increases seen in Vitamin E and cholesterol proved to be statistically significant

5 Although the phospholipid levels of the supplemented patients were 10.4 and 18.5 per cent higher than those of the controls at twenty four and thirty six months, respectively, only the thirty six month figure proved to be statistically significant

6 Assays made six months after high vitamin A supplementation was discontinued showed that the previously indicated elevations of vitamin E, cholesterol, and phospholipid had returned to levels not statistically different from the controls

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# A COMPARATIVE STUDY OF MICRO AND MACROELECTROPHORETIC ANALYSIS OF HUMAN AND RAT SERUM

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A DETAILED comparative study of electrophoretic analysis of protein solutions using a microcell (2 ml capacity) and the standard macrocell (11 ml capacity) has not been reported previously. Ross, Moore and Miller<sup>1</sup> published a study on human seminal plasma in which a microcell was used. They stated that the mobilities and character of the patterns obtained agreed exactly with those observed with the 11 ml cell. Comparative data, however, were not reported.

Since the length of the channel of the microcell is only five ninths that of the macrocell, the time of electrophoresis becomes a limiting factor when proteins with relatively high mobilities, such as albumin, are being studied. Thus, in the case of serum, complete resolution of the components will be dependent in part upon the migration distance of the albumin component in the microcell. Aside from this, there is no theoretic basis for anticipating differences in electrophoretic analysis with the two cells. However, the fact that the two cells are different in design and manipulation would make a detailed comparative study desirable. The great value of having available a microcell for electrophoresis of small samples is obvious.

## METHODS AND PROCEDURES

### *Use of the Microcell —*

The microcell used in this study is a commercially available model and has the following dimensions: capacity, 2 ml, length of center section 5 cm, cross sectional area 0.30 centimeter. The cell and the microelectrode vessels are mounted in a special rack adapted for the micro assembly. The center section of the microcell is moved by a pair of sliding racks and pinions, as with the macroassembly.

Since the commercially available microcells are open on both sides a few modifications of the usual technique are necessary. In the first place, it is necessary to lower the water level of the bath to a point just below the top of the microcell. Since with the usual low temperature bath arrangement this will leave a portion of the cooling coil exposed it is necessary to lower the coil to a point where it is completely immersed at the lower water level. It is obvious that unless this is done the coil will ice and freeze the stirrer. It is desirable when lowering the coil to arrange for a pair of oppositely pitched paddles on the stirrer to aid in circulating water at the top and bottom of the coil. Special masks with slits adapted to the microcell dimensions must be used. The mask over the schlieren lens and the slit mask used in scanning are modelled after the macromasks. It was found convenient to solder a pair of piano wires across the inner surface of the slit mask toward the top and bottom to serve as reference lines.

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Obtainable from either the Ilett Manufacturing Co. New York N Y or the Pyrocell Manufacturing Co. New York N Y.

It is possible to close off one side of the microcell by the use of a close fitting lucite plug inserted into the upper sections. In this way the microcell can be used in much the same manner as the macrocell. If this is attempted the electrode is set on the closed side must of course be fitted with a three way stopcock etc.

The microcell is set up in essentially the same manner as is the larger cell.<sup>2</sup> The electrode vessels are attached and filled with particular care to remove entrapped air bubbles from the small side arms. The cell carrier is placed in the thermostat, saturated salt solution is added to surround the electrodes, and the whole system is allowed to equilibrate. Before aligning the cell, it has been found necessary to remove the excess buffer from the bridge between the two channels of the top section. This is most readily effected by blotting with a piece of filter paper. Because of differences in the specific gravity of the protein and the buffer solutions on the two sides of the cell, it is advisable to equalize the hydrostatic pressure on the two sides by removing a small amount of buffer from the top section of the protein side before aligning the cell. This serves to prevent a rapid shift in the position of the boundaries once they are formed. As a convenient method for bringing the boundaries into view, one end of a piece of fine glass capillary is attached by means of rubber tubing to the compensating syringe and the drawn out end is inserted directly into the top section of the cell on the ascending (anode) side. The compensator gears are then reversed, whereupon a slow and regular withdrawal of fluid takes place.

Once the boundaries are in view, the starting position and base line pictures can be taken. Electrophoresis is then carried out in the usual manner except that the voltage is reduced so as to maintain approximately the same potential gradient of 5 to 6 volts per centimeter. Because of the smaller cross sectional area of the microcell, a current of 8 Ma was maintained throughout the run, rather than the 15 Ma used with the macrocell. Under these conditions the electrophoresis time for serum is limited to about ninety minutes because of the short length of the center channel of the microcell.

In all instances serum samples were diluted with two parts of buffer before dialysis.

## RESULTS

In Table I are listed values obtained with ten different pathologic human sera using both the micro- and macrocell. As can be seen, the correlation co

TABLE I COMPARISON OF MACRO AND MICROELECTROPHORETIC ANALYSIS OF PATHOLOGIC HUMAN SERUM

NO	PER CENT COMPOSITION									
	ALBUMIN		ALPHA-1		ALPHA-2		BETA		GAMMA	
	MACRO	MICRO	MACRO	MICRO	MACRO	MICRO	MACRO	MICRO	MACRO	MICRO
1	29.4	31.4	14.4	12.6	15.2	12.9	15.6	23.8	25.4	19.3
2	26.4	30.2	8.9	8.5	16.4	17.7	24.6	23.8	23.7	19.8
3	46.1	49.0	12.5	10.5	16.2	14.3	14.5	15.9	10.7	10.3
4	31.4	30.4	15.9	17.5	19.2	17.5	18.0	22.9	15.5	11.8
5	40.3	45.3	9.7	7.9	19.7	19.6	17.9	16.8	12.4	10.4
6	44.4	46.8	13.1	13.2	12.3	12.4	17.4	17.7	12.8	9.9
7	51.4	49.9	9.8	9.9	10.0	10.2	16.1	17.8	12.7	12.2
8	23.3	24.8	11.3	11.5	13.4	10.9	20.1	22.6	31.9	30.2
9	42.7	42.7	8.7	8.6	12.4	12.7	19.9	20.5	16.3	15.5
10	52.4	51.4	6.8	6.2	9.8	9.1	20.6	23.3	10.4	10.0
Mean	38.8	40.2	11.0	10.6	14.5	13.7	18.5	20.5	17.2	14.9
S.D.	9.8	9.4	2.7	3.1	3.30	3.33	2.79	2.94	6.9	6.2
r	+0.987		+0.850		+0.920		+0.575		+0.975	
p	< 01		< 01		< 01		< 05		< 01	

Diagnosis of cases as follows: 1 nonlipoid histiocytosis (Letterer-Siwe's disease); 2 carcinoma of esophagus with metastases; 3 agranulocytosis with terminal lobar pneumonia; 4 angiomatous mesothelioma of pelvis; 5 localized Hodgkin's disease with infiltration of lungs; 6 arteriosclerosis generalized; 7 myelogenous leucemia; 8 hypernephroma with metastases; 9 hypertensive and arteriosclerotic heart disease with multiple pulmonary emboli; 10 broncho pneumonia. Electrophoretic analyses of cases 1 to 9 have been previously reported.<sup>3</sup>

$$r = \text{Correlation coefficient} = \frac{\sum x_i y_i}{\sqrt{\sum x_i^2 \sum y_i^2}} \text{ where } \bar{x} = \frac{\sum x}{n} \text{ and } \bar{y} = \frac{\sum y}{n}$$

$$s_x = \text{Standard deviation (S.D.) } s_x = \sqrt{\frac{\sum x^2}{n} - \bar{x}^2}$$

p = Probability of chance variation obtained by use of r values and a table of probabilities. p value of < 05 indicates the probability of chance variation of less than 5 in 100. p value of < 01 indicates chance variation of less than 1 in 100.

efficients are high in all instances except in the case of the beta globulin values. The known difficulty of accurately defining and measuring the beta globulin area on the descending side in all probability accounts for the less satisfactory but still significant correlation of these values.

In Table II are listed two sets of values taken from the same individual. The microdeterminations represent three different samples taken within a period of one month, the macrodeterminations represent triplicate determinations on a single sample taken from the same individual one year later. It is apparent that the range of variation within the two sets of determinations is of the same order in both the micro and the macro runs.

TABLE II COMPARISON OF MACRO AND MICROELECTROPHORETIC ANALYSIS OF NORMAL HUMAN SERUM

NO	PER CENT COMPOSITION				
	ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
<i>Macro</i>					
1	61.2	5.0	8.1	13.8	11.9
2	60.2	6.2	8.2	12.2	13.2
3	61.0	5.6	7.6	12.4	13.4
Average	60.8	5.6	8.0	12.8	12.8
<i>Micro</i>					
4	65.7	4.0	7.0	12.5	10.8
5	63.8	4.6	7.7	12.0	11.9
6	63.6	5.0	7.8	11.3	12.3
Average	64.4	4.5	7.5	11.9	11.7

In Table III the per cent composition of normal rat serum using the micro and macrocell is shown. The mean values and standard deviations of the two sets of data are of the same order of magnitude. While these data do not represent duplicate determinations, one micro and the other macro, on the same

TABLE III COMPARISON OF MACRO AND MICROELECTROPHORETIC ANALYSIS OF NORMAL RAT SERUM

NO	PER CENT COMPOSITION				
	ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
<i>Macro</i>					
1	50.0	18.0	13.8	12.1	6.1
2	51.4	18.0	10.4	16.2	4.0
3	49.8	16.4	11.1	17.6	5.1
4	52.8	17.7	8.9	17.0	3.6
5	50.4	20.7	9.4	13.6	5.9
6	49.3	21.1	10.2	15.5	3.8
Mean	50.6	18.6	10.6	15.4	4.8
S.D.	1.05	1.68	1.45	1.92	1.00
<i>Micro</i>					
1	48.4	19.1	13.0	14.0	5.5
2	46.9	20.5	10.8	17.3	4.5
3	53.4	16.5	9.7	15.2	5.2
4	50.5	16.7	11.0	15.9	5.9
5	50.6	16.4	10.4	18.4	4.2
6	49.4	17.7	7.2	18.3	7.4
	51.7	15.9	7.3	17.5	7.6
Mean	50.1	17.5	9.9	16.6	5.7
S.D.	1.54	1.56	1.93	1.54	1.22

serum, they indicate that in a series of determinations using normal rat serum the percentage compositions will be comparable whether determined with the micro- or the macrocell

A comparison of mobility values obtained with the micro- and macrocell with rat and human serum is shown in Table IV. While the mean values are in reasonably close agreement, it is of interest that the standard deviations in the case of the rat serum macrodeterminations are considerably higher than those for the microdeterminations. In all probability a larger series of samples would not show this.

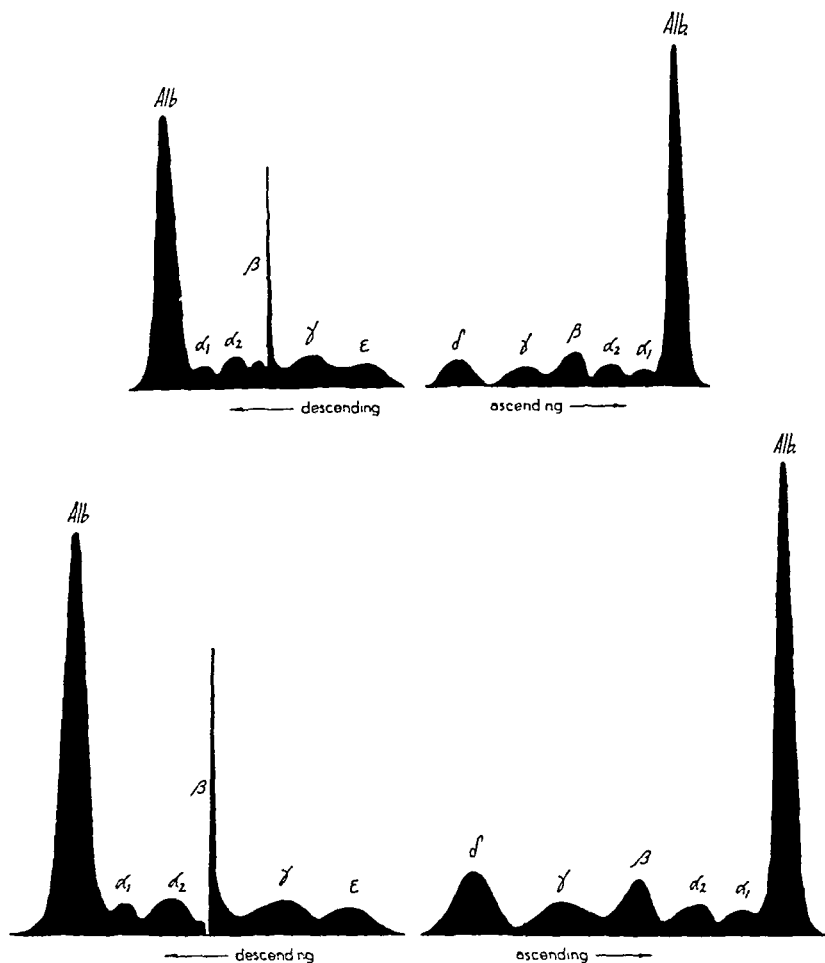


Fig. 1—Comparison of macro- and microelectrophoretic patterns of normal human serum. Electrophoresis time: macro 150 minutes; micro 90 minutes. Protein concentration 2 Gm. per 100 milliliters. Relative dimensions of patterns are maintained.

In Fig. 1 tracings of a macro- and a microelectrophoretic pattern of normal human serum from the same individual are reproduced. It is apparent that resolution after 90 minutes with the microcell compares favorably with that of the macrocell, run for 150 minutes.

TABLE IV COMPARISON OF MOBILITY VALUES OBTAINED WITH MACRO AND MICROCELLS

	MOBILITIES $\times 10^5$ (CM PER VOLT SECOND)									
	ALBUMIN		ALPHA-1		ALPHA-2		BETA		GAMMA	
	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.	MEAN	S.D.
Human										
micro	6.7	0.35	5.6	0.27	4.7	0.22	3.2	0.25	1.3	0.26
macro	6.7	0.33	5.7	0.32	4.5	0.30	3.2	0.30	1.4	0.32
Rat										
micro	6.1	0.17	5.4	0.12	4.5	0.20	2.9	0.11	1.7	0.15
macro	6.1	0.32	5.4	0.37	4.3	0.35	2.9	0.23	1.7	0.31

In Fig. 2 tracings of a macro and a microelectrophoretic pattern of two different normal rat sera are shown. It is to be noted that while rat serum does not show the ready resolution seen with human serum the macro and micro patterns are quite comparable.

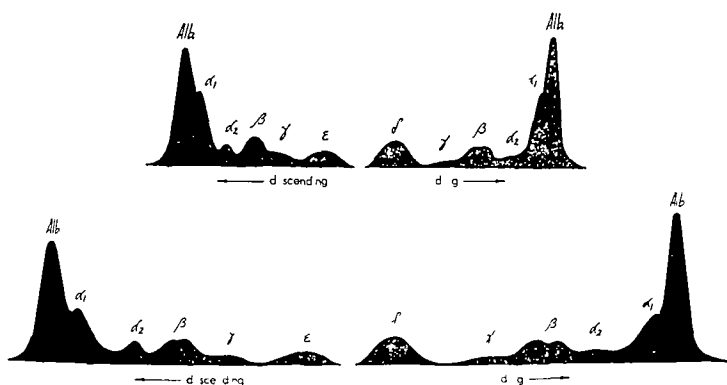


Fig. 2.—Comparison of macro and microelectrophoretic patterns of normal rat serum. Electrophoresis time: macro 180 minutes; micro 90 minutes. Protein concentration 2 Gm. per 100 milliliters. Relative dimensions of patterns are maintained.

#### DISCUSSION

The comparative data for macro and microelectrophoretic analyses of serum proteins reported here indicate that the values obtained by the two methods are in close agreement. In instances in which one is dealing with samples of serum of small volume the practical importance of the microcell is apparent. However, it is important to emphasize that a series of comparative determinations of the type reported here may be required in any study in which the two cells are to be used particularly when quantitative differences of small magnitude are anticipated. It should also be pointed out that protein mixtures other than serum have not been extensively studied and therefore preliminary investigation of completeness of resolution of the component proteins must be established beforehand.

## SUMMARY

1 Electrophoretic patterns of normal and pathologic human serum and normal rat serum have been compared using a micro- and a macroelectrophoresis cell. The mobilities and per cent composition of the components are in close agreement by the two methods.

2 Some technical aspects of the use of the microcell are presented and discussed.

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# STERNAL MARROW HEMOSIDERIN

A METHOD FOR THE DETERMINATION OF AVAILABLE IRON STORES IN MAN

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**A** KNOWLEDGE of the available iron stores is useful in the management of anemia. The extreme manifestations of iron lack such as koilonychia, glossitis, dysphagia, and microcytic hypochromic red cells are the result of a long existing deficiency. Iron excess is even more difficult to recognize than its lack, except in cases of hemochromatosis or severe hemosiderosis where there are pigmented deposits in the skin or in the urinary sediment. The majority of clinical problems in iron metabolism lie between these extremes, and in this group the examination of sternal marrow has proved to be a convenient and reliable index of iron deficiency or iron excess.

## MATERIAL AND METHODS

One or more sternal punctures were performed on sixty-three individuals. This group included eleven normal subjects, sixteen patients with iron deficiency anemia, eleven with pernicious anemia, six with uremia, five with ulcerate or chronic infection, five with cirrhosis of the liver, three with lupus erythematosus, two with hemochromatosis, and four with hemosiderosis secondary to multiple transfusions. Only clear cut examples were selected for this report in which there was a careful history relevant to blood loss and iron intake by mouth or parenterally. Patients with iron deficiency anemia showed typical microcytosis and hypochromia which were associated with a low serum iron in the patients in whom the serum iron was determined. The diagnosis of pernicious anemia was confirmed by the characteristic cell indices and blood cytologic changes, megaloblastic sternal marrow, and a satisfactory reticulocyte response to intramuscular liver. Those with uremia presented the clinical picture of severe renal damage with a blood urea nitrogen in the neighborhood of 100 mg. per cent. Patients in the infectious group had a daily oral temperature of 100 F. and above for more than two weeks. All the patients with cirrhosis had obvious impairment of function tests and other stigmata of chronic liver disease. Both patients with hemochromatosis presented the typical hepatic lesions of that disease by biopsy and had a high serum iron with complete saturation of the iron binding protein characteristic of hemochromatosis.<sup>1</sup> The four patients with extensive hemosiderosis had had twenty-eight, eleven, fifty, and forty-nine transfusions respectively.

Sternal punctures were performed ‡. After preliminary novocain infiltration a 2½ cm. No. 14 needle with a short bevel is introduced into the sternal marrow cavity in the midline at the level of the second interspace. The stylet is then withdrawn and 4 c.c. of marrow and blood are drawn into a 20 c.c. syringe containing 6 c.c. of 4 per cent sodium citrate. This mixture is then ejected into a large watch glass and cover slip films are made from marrow fragments picked up with a capillary pipette.

The preparations are examined microscopically both unstained and after staining with hydrochloric acid and ferrocyamide (Berlin blue stain).§ In the unstained preparations the hemosiderin appears as golden yellow granules under reduced illumination. In the stained preparation the granules take a blue stain. Not infrequently structures other

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¶According to the technique employed by Dr. Joseph Ross.

§The stain was prepared as follows: 4 Gm. of potassium ferrocyanide were diluted to 20 c.c. with water. Concentrated hydrochloric acid was added until a white precipitate formed. This was filtered and the smears covered with the filtrate for thirty minutes.



than hemosiderin will stain, usually in proportion to the amount of iron present. These artifacts can be recognized with experience and by comparing the two types of preparation. In iron deficiency, pale yellow granules which do not take iron stain are sometimes seen. These may be analogous to the protein granules of hemosiderin remaining after removal of iron by extraction with 10 per cent hydrochloric acid.<sup>2</sup> The pale yellow color is probably due to adsorbed bilirubin. The smears were graded according to the amount of iron present.

0	None	4	Moderately heavy
1	Very slight	5	Heavy
2	Slight	6	Very heavy
3	Moderate		

## RESULTS

In Fig 1, examples of these marrow preparations are demonstrated. Each marrow was graded independently by each of us and there was close agreement in most of the material examined. The independent ratings rarely varied more than one grade in either direction.

In the normal group the marrow iron was graded as 1 or 2 in every case except one which received a grade of 3 (Table I). All of the patients with

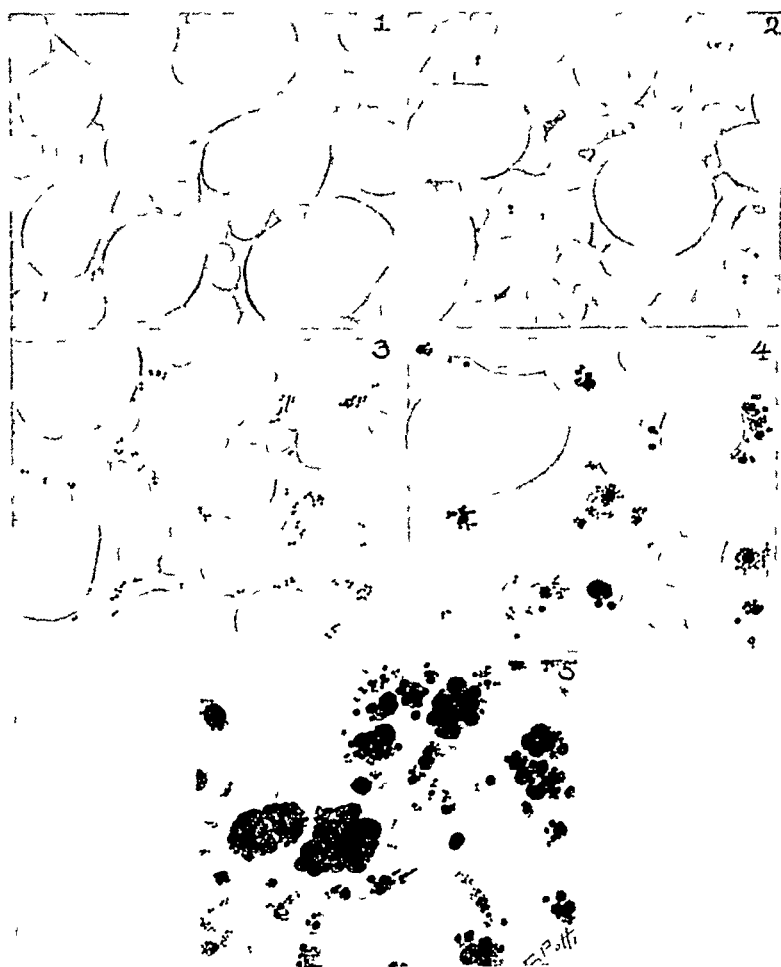


Fig 1.—Sternal marrow preparations in which the hemosiderin deposits are illustrated as black granules. 1 Grade 0 patient with severe iron deficiency. 2 Grade 2 normal subject. 3 Grade 3 patient with pernicious anemia. 4 Grade 4 patient with chronic infection. 5 Grade 6 patient with multiple transfusions.

TABLE I NORMAL SUBJECTS

SUBJECT	SEX	AGE	HB (GM)	MCV* (CU $\mu$ )	MCH† (γγ)	MCHC‡ (%)	DIAGNOSIS	STERNAL MARROW IRON
W M	M	25	15.6	89	31	35	Normal	2
C O	M	24	15.8	88	30	33	Normal	1
C F	M	32	16.0	82	28	34	Normal	1
A M	M	29	17.5	91	32	39	Normal	2
P McD	M	21	13.8				Acute pharyngitis	2
G T	M	48	19.2				Obesity	3
J F	M	30	15.0				Cholelithiasis	2
F W	F	44	14.7				Dermatitis	2
E H	F	56	15				Parkinsonism	1
J M	M	61	16.9				Hypertensive cardiovascular disease	2
M S	M	62	16.4				Generalized arteriosclerosis cerebral arteriosclerosis	2

Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

TABLE II IRON DEFICIENCY ANEMIA

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† (γγ)	MCHC‡ (%)	SOURCE OF BLOOD LOSS	STERNAL MARROW IRON
J C	39	F	5	54	14	25	?	0
R C	28	F	12.5				Thrombocytopenic purpura with menorrhagia	0
T B	68	F	7.9	68	19	28	Carcinoma of colon	0 1½
J D	47	M	7.2	63	16	26	"	0
M H	77	F	6.8	63	17	26	Achlorhydria	0
N F	14	F	9.6	75	25	31	"	0 1½
V C	18	F	13.4	71	22	31.5	"	0 1½
A Y	3	F	5.5	65	18	27	"	0 1½
J M	46	M	8.5	98	29	30	Acute bleeding ulcer	0
R N	6	M	8.2	88	30	35	Acute bleeding ulcer	0 1½
M M	35	F	9.0				Idiopathic thrombocytopenia	0
E I	56	M	10.1	82	23	29	Gastrointestinal bleeding	1½
M B	15	F	10.6				Idiopathic thrombocytopenic purpura	0 1½
J S	77	M	9.7				Hiatus hernia	0
E McL	62	F	7.8	71	18	25	"	0
A B	27	F	5.5	60	14	24	"	0

Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

§Those with sternal marrow iron grade 1 showed pale yellow granules which stained little or not at all with the iron stain

TABLE III PERNICIOUS ANEMIA

SUBJECT	AGE	SEX	HB (GM)	MCV (CU $\mu$ )	MCH† (γγ)	MCHC‡ (%)	STERNAL MARROW IRON
P A	58	F	10.2	112		4	2
M McM	71	M	5.8	126	4	4	4
K MacL	54	M	11.2	106	37	35	4+
M G	51	F	7.9				+
H D	32	F	6.4	100	36	36	3
M I	77	M	5.1	115	40	36	5
M P	69	F	8.0	99	38	38	5
H P	68	M	8.5	115	45	34	5
V A	58	F	9.1	100	6	36	2+
C C	56		8.0	110	36	3	4+
J P	76	F	12.7	118	42	35	0 1

Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

TABLE IV URFEMIA

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† ( $\gamma\gamma$ )	MCHC‡ (%)	DEGREE OF UREMIA	STERNAL MARROW IRON
B R	46	F	7.6	90	27	31	BUN    113§	0.1
B A	67	F	4.7				BUN 110	0.1
Z A	56	F	11.0	72	26	36	BUN 50	0
B B	30	M	11.0				BUN 103	4+
A M	79	M	8.5				BUN 90	3
G G	49	M	6.5	82	26	32	BUN 190	2+

\*Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

§Recurrent epistaxis

||Blood urea nitrogen

TABLE V CIRRHOSIS

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† ( $\gamma\gamma$ )	MCHC‡ (%)	BLOOD LOSS	STERNAL MARROW IRON
H F	64	M	10.5	109	34	31	None known	4
G McG	62	F	7.4	109	36	32	None known	4
A W	51	F	7.5	102	29	30	Gurgle positive stools	0.1
D M	64	M	12.0				None known	3
J W	69	M	10.7	124	41	33	None known	1.2

\*Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

TABLE VI INFECTION

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† ( $\gamma\gamma$ )	MCHC‡ (%)	DIAGNOSIS	STERNAL MARROW IRON
N D'Q	25	F	9.7	85	29	36	SBE§ six months	3.4+
J I	60	F	9.4	97	33	32	Chronic pul monary disease, five years	4+
C J	65	M	11.0	92	29	31	Severe pneu mococcal pneumonia	5
S D	42	M	13	87	28	33	SBE three months	3+
H S	54	F	9.5	79	25	32	PUO   two months	3+

\*Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

§Bacterial endocarditis

||Fever

TABLE VII DISSEMINATED LUPUS ERYTHEMATOSUS

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† ( $\gamma\gamma$ )	MCHC‡ (%)	STERNAL MARROW IRON
N McG	39	F	8.3	92	27	30	4+5-
D S	39	F	5.9	92	29	32	5
E R	19	F	9.7				5

\*Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

iron deficiency anemia showed a marrow iron of 0 or 1 (Table II). Of the eleven patients with pernicious anemia only one showed a marrow iron of less than 3, and five of the eleven showed a marrow iron of 4 or more (Table III). In the patients studied, the granules presented a somewhat characteristic appearance in that they were small, numerous and of uniform size (Fig 1-3). In the patients with uremia the sternal marrow iron ranged from 0 to 4 plus (Table IV). The cirrhotic patients showed a normal or increased iron in three of five patients (Table V). Five patients with infection showed an increase in the marrow iron (Table VI). Three patients with lupus erythematosus showed a heavy marrow iron deposition (Table VII). The heaviest iron deposition, grades 5 and 6, was seen in the patients with hemochromatosis and hemosiderosis (Tables VIII and IX).

TABLE VIII HEMOCHROMATOSIS

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† ( $\gamma\gamma$ )	MCHC (%)	STERNAL MARROW IRON
E M	58	M	15.2	96	31	32	4+
M B	62	M	11.8	94	23	25	5+

\*Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

TABLE IX HEMOSIDEROSES

SUBJECT	AGE	SEX	HB (GM)	MCV* (CU $\mu$ )	MCH† ( $\gamma\gamma$ )	MCHC‡ (%)	NUMBER OF TRANSFUSIONS	STERNAL MARROW IRON
A A	56	M	5.5	93	27	29	28	5
H K	66	M	7.6	115	38	33	11	5
J S	55	M	6.4	100	34	34	50	5
P M	23	F	5	101	32	32	3 (at onset of anemia)	2+
	25	F	10.0	91	29	32	49 (18 months later)	6+

\*Mean corpuscular volume

†Mean corpuscular hemoglobin

‡Mean corpuscular hemoglobin concentration

## COMMENT

Storage iron in man exists as ferritin and hemosiderin. The precise relationship between them is not clear. It would appear however that ferritin represents a labile or active form of iron storage and that hemosiderin is a less active form of iron storage present only when there are adequate ferritin stores.<sup>2</sup> The studies of Boggs<sup>3</sup> and Whipple<sup>4</sup> have demonstrated that hemosiderin is mobilized for hemoglobin production when needed by the body. Of these two forms of iron storage hemosiderin is the only one morphologically identifiable. These yellow or brownish yellow granules are found throughout the reticuloendothelial system and macrophages of the body. The sternal marrow provides a readily accessible portion of the reticuloendothelial system for biopsy.

In this group of patients the sternal iron has been consistent with the alteration of iron storage characteristic of these diseases. In infection there was

an increase in hemosiderin above the amount normally seen. The progressive hemosiderosis in this condition appears to be due to an increased affinity of the tissues for iron. In pernicious anemia there is likewise an increase in tissue iron due in part at least to storage of red cell iron in the tissues. In hemochromatosis, sternal iron is increased as are other iron stores throughout the body. Multiple transfusions provide even heavier deposits of iron since the iron provided by the donated red cells is not excreted in appreciable amounts from the body. Patients with cirrhosis and nephritis have variable iron stores due to the variable bleeding in these diseases. Iron deficiency, in contrast to the normal controls and the other diseases described, shows an absence of iron staining granules. In this group of patients subsequent iron therapy was effective in alleviating the anemia.

It is to be expected that the balance of blood loss and iron absorption over a period of years will vary considerably from person to person, although this is remarkably well regulated by the absorptive mechanism.<sup>6</sup> However, certain conditions will modify the stores to an extent which overshadows these normal variations. That a clear-cut differentiation of the anemia of infection from that of iron deficiency usually may be made is evident from comparing Table II and VI. Hemochromatosis also may be excluded in patients with cirrhosis if the marrow iron is not increased (Tables V and VIII).

A number of patients referred to us with mild anemias reputed to be iron refractory were shown by marrow puncture to have adequate or increased iron stores. Those anemias were felt to be due to other causes, that is, obscure infection or damaged bone marrow. In our experience, only patients with absent marrow iron will be benefited by iron therapy.

#### SUMMARY

A method of estimating tissue iron stores by sternal puncture has been described.

A group of sixty-three patients have been studied and the hemosiderin content of the marrow was found to parallel the anticipated iron storage in these diseases.

The presence or absence of iron in the marrow may be regarded as an index of the need for iron therapy in anemia.

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## OCCURRENCE OF TOXOPLASMA NEUTRALIZING ANTIBODIES IN VARIOUS DISEASE CONDITIONS

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THE toxoplasma neutralization test has been used extensively as an aid in the diagnosis and recognition of nonfatal and inapparent infection in man. With this procedure or the unequivocal demonstration of parasites in tissues either by animal inoculation or by histologic methods it has been found that toxoplasmic infection can be responsible for many conditions such as (1) congenital encephalomyelitis, which becomes manifest either in utero or shortly after birth (2) acute encephalitis in children, (3) infection in adults resembling Rocky Mountain spotted fever, and (4) mild or inapparent infection. An investigation of the occurrence of toxoplasma neutralizing antibodies in the blood of certain selected individuals is the basis of this report.

The method for performing the neutralization test has been reported in detail by Sabin<sup>1</sup> and consists of the following procedure with minor modifications. A freshly prepared 10 per cent toxoplasma infected mouse brain suspension in saline is allowed to sediment spontaneously for one half hour. The supernatant fluid is drawn off and further dilutions of 1:50, 1:500 and 1:5,000 are made. Equal volumes of these dilutions (0.15 cc) are added to undiluted serum (or Tyrode's solution for the controls) giving final dilutions of 1:20, 1:100, 1:1,000, and 1:10,000. The mixtures are incubated at room temperature for approximately one half hour at which time 0.2 cc amounts are injected intracutaneously on the back of a rabbit. The lesions which develop are measured at the end of seven days. The inhibition of skin lesions by the test sera are compared with control lesions and the degree of neutralization determined. The criteria for interpretation of results are those advanced by Sabin.<sup>1</sup>

Serum specimens were kept as fresh as possible and either used immediately or stored in dry ice until needed. They were obtained from individuals who along with other signs presented one or more of the following manifestations: hydrocephalus or microcephaly, cerebral calcification, chorioretinitis or other eye changes, and involvement of the central nervous system. Members of the immediate families were also examined for the presence of antibodies wherever indicated. Tests were also performed on patients with diseases of unknown etiology in order to determine a possible relationship to toxoplasmosis. Individuals suspected of having a congenitally acquired illness were also tested.

The results of the ninety tests performed with the sera from seventy-two individuals are listed in the following tables. Reported in Table I are positive results on seven patients, one month to five years of age, who presented evidence

<sup>1</sup> From the Children's Hospital Research Foundation and the Department of Bacteriology and Pathology of Cincinnati College of Medicine.  
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of having acquired the infection in utero, since they were either sick at birth or became sick shortly after birth. Six of the patients in this group had cerebral calcification, five had chorioretinitis or other signs of eye involvement, three had hydrocephalus, one had microcephaly, and six manifested convulsions or other signs of central nervous system involvement. Additional findings were a rash in two patients and splenomegaly in one patient. It is now apparent that cerebral calcification and central chorioretinitis are the important signs of congenital toxoplasmosis. One or the other or both were present in six of the patients. The seventh showed only microcephaly and mental retardation. In each instance tests of the mothers' sera showed toxoplasma neutralizing antibodies, further proof of the congenital inception of the disease. The father and an older sibling of one of the patients also developed antibodies. It is noteworthy that the serum from patient Bla,<sup>4</sup> which was positive at the age of one year, still showed the presence of antibodies when tested more than five years

TABLE I PATIENTS WHOSE SERA NEUTRALIZED TOXOPLASMA

INCEPTION	PATIENT	AGE	CHIEF FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Gri †	1 mo	Cerebral calcification, hydrocephalus, chorioretinitis, convulsions, rash	[M] [F] [S] ⑥
	Cle	1 mo	Cerebral calcification, convulsions, head rolling, apnea, xanthochromia	[M]
	Seu	5 mo	Microcephaly, mental retardation	[M]
	Gro	6 mo	Cerebral calcification, rash, splenomegaly, retrolental fibroplasia	[M]
	Kam	2 yr	Cerebral calcification, hydrocephalus, chorioretinitis, eyes of different size	[M] ⑥ ⑥
	Kuh ‡	4 yr	Cerebral calcification, chorioretinitis, convulsions, mental retardation	[M]
	Bla §	5 yr	Proved case, 5 years later cerebral calcification, hydrocephalus, chorioretinitis	[M]
Probably congenital	Kre	7 yr	Chorioretinitis, psychomotor disturbances	--
Acquired	Lyk	9 yr	Encephalitis last six weeks, questionable brain tumor	⑥
	Jaq	32 yr	Mental disturbance (psychoneurotic)	--
	Par	62 yr	Encephalitis and mental disturbance for one week	--

\*[ ] Neutralized ○ did not neutralize M mother F father S sibling -- not available

†Proved fatal case

‡Case reported by Miller<sup>2</sup>

§Case reported by Sabin<sup>4</sup>

later. Inoculation of spinal fluid and blood from two of the patients (Kam and Lyk) into mice was without effect. However, toxoplasma were readily recovered from animals inoculated with ground suspensions of tissues obtained from patient Gri at autopsy. Positive tests on the sera of four other patients (7 to 62 years of age) are also reported in Table I. In these patients the onset of the illness could not be determined with certainty. Patient Kre, 7 years of age, showed unilateral chorioretinitis (type not determined) and psychomotor

disturbances. The mother was not available for testing. Patient Lyl, 9 years of age whose serum was positive, had had encephalitis for the previous six weeks. A retest several months later was again positive while the mother's serum was negative. Included in this series are two adults: one, a 32 year old female psychoneurotic patient with a questionable optic atrophy, and the other a 62 year old male who was said to have had encephalitis of about one week's duration from which he completely recovered. Two months previously he had been bitten by a cat that later died in convulsions. One cannot assume, however, that toxoplasma were the cause of these conditions because these patients might have acquired the antibody sometime in the past as a result of inapparent infection.

TABLE II PATIENTS WITH HYDROCEPHALUS WHOSE SERA FAILED TO NEUTRALIZE TOXOPLASMA

INCEPTION	PATIENT	AGE	ADDITIONAL FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY
Congenital	Car †	1 mo	Macular chorioretinitis psychomotor disturbance	--
	Kle	3 mo	Difficult delivery transitory nystagmus	(M)
	Mad	6 mo	Birth injury subdural hematoma spasticity	--
	Dam	7 mo	Mental retardation nystagmus tumor mass in right eye	(M)
	Aut Lem	1 yr 2½ yr	Meningocele at birth Difficult delivery mental retardation	--
Probably congenital	Tav	5 mo	Mental retardation	(M)
	Mil	18 mo	Congenital heart disease mental retardation	--
	Rus	20 mo	Cerebral atrophy ataxia strabismus	(M)
	Lit	3½ yr	Onset at 1 year chorioretinitis mental retardation convulsions	(M) (S) (S)

○ Did not neutralize M mother S sibling -- not available

† Patient too young to develop own antibodies

As recorded in Table II the sera of ten children with hydrocephalus (one month to three and one half years of age) were completely negative for toxoplasma neutralizing antibodies. In most instances the hydrocephalus resulted from developmental defects or trauma but in two patients only the hydrocephalus was associated with chorioretinitis. Patient Car had chorioretinitis in the macular region of both eyes and hydrocephalus at the age of 1 month. It should be pointed out, however, that the neutralization test may be negative in a newly born infant and positive when the child is several months old. Thus it is of greater importance to test the mother's blood as early as possible after the birth of the baby, and the child's blood several months later. Unfortunately in this case neither the child nor the mother was available for subsequent tests. Patient Lit, with a possible onset at one year of age developed hydrocephalus convulsions and chorioretinitis whose type and distribution was not determined. In this instance the sera of the child and the mother were negative. It is worth noting that in the series of children without antibodies the mothers' sera were also found to be negative. The instances in which the sera of both mother and



child are positive therefore assume greater significance as serologic evidence for a diagnosis of congenital toxoplasmosis

Cerebral calcification in the absence of toxoplasma neutralizing antibodies was encountered four times (Table III). In two of the patients (Fle and Ste) the calcification was associated with developmental defects. However, toxoplasmosis could not be excluded with certainty in two (Bel and Nei), one patient in particular (Bel) having manifestations compatible with a diagnosis of toxoplasmic infection. The sera from the mother and child were sent through the regular mail during the hot summer months without any precautions for preserving the heat-labile toxoplasma antibody. Even so, they were not completely negative and it is highly probable that antibodies might have been demonstrated in a fresh specimen. Further specimens were not obtainable. Patient Nei also gave a history compatible with a diagnosis of toxoplasmosis, but repeated tests many months apart failed to reveal neutralizing antibodies in either the mother or the child. The absence of neutralizing antibodies in a child showing the clinical signs of congenital toxoplasmosis has been reported.<sup>1</sup> Both the mother and child failed to show toxoplasma neutralizing antibodies on repeated tests but regularly showed the presence of complement-fixing antibodies. Again in this group the mothers as well as the offspring lacked the neutralizing antibodies.

TABLE III PATIENTS WITH CEREBRAL CALCIFICATION WHOSE SERA FAILED TO NEUTRALIZE TOXOPLASMA

INCEPTION	PATIENT	AGE	ADDITIONAL FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Fle	1½ mo	Spina bifida, meningocele	(+) (Equivocal)
	Ste	16 mo	Failure to develop	(+) (Equivocal)
	Bel†	3 yr	Bilateral macular chorioretinitis, micro cornea	(+) (Equivocal)
	Nei	4 yr	Microcephaly, mental retardation, optic atrophy	(+)

Zone of calcification: Fle, right hemisphere between parietal bone and right coronal suture. Ste, frontoparietal area near left vertex. Bel, linear calcifications in the brain. Nei, walls of ventricles.

\*O Did not neutralize. M, mother.

†Serum specimens were in the mail for several days during the summer months. Results of the neutralization test were equivocal.

Negative results were obtained with the blood of six patients, 2 months to 27 years of age with retinal lesions but without signs of hydrocephalus or cerebral calcification (Table IV). In addition to other symptoms, two members of the group, age 2 and 5 months, respectively, developed convulsions. The 5-month-old child had central choroiditis and nystagmus as well. Serum tests of the mothers of these children were likewise negative for toxoplasma antibodies. The remaining four patients were of sufficient age to develop antibodies but, since none were found, no attempt was made to examine the mothers' blood. The type and distribution of lesions in these individuals as well as the possible

TABLE IV PATIENTS WITH EYE CHANGES WHOSE SERA FAILED TO NEUTRALIZE TOXOPLASMA

INCEPTION	PATIENT	AGE	FINDINGS	RESULTS OF NEUTRALIZATION TESTS WITH SERA FROM IMMEDIATE FAMILY*
Congenital	Mol	2 mo	Eyes closed, slight retinal changes convulsions	(M)
	Ans	5 mo	Bilateral macular chorioretinitis, nystagmus convulsions	(M)
	Hil	3½ yr	Inability to develop since birth chorioretinitis (2 years later)	--
Probably congenital	Gai	2 yr	Bilateral chorioretinitis (diffuse pigmented)	--
Acquired	Glo†	19 yr	Active bilateral macular chorioretinitis	-
	Rom	27 yr	Bilateral chorioretinitis (hemorrhagic) in last year	-

\*O. Did not neutralize M mother — not available

†Equivocal on repeated tests

onset are listed in Table IV. The chorioretinitis was first noted about two years after birth in patient Hil. In the adults, Glo and Rom, the chorioretinitis was active and had developed within the past year.

The determination of neutralizing antibodies was extended to include disease conditions in which a definite etiology could not be established. When individuals with signs of hydrocephalus, cerebral calcification and chorioretinitis had been eliminated there was still a large group (ages 7 months to 74 years) that showed negative neutralization tests. In many instances (especially in children with congenitally acquired diseases) the tests were performed to exclude the possibility of toxoplasmosis. The primary diagnosis or chief findings are listed in Table V. In this group three patients had prolonged encephalitis, six had encephalopathies of obscure etiology, three were believed to have Hodgkin's disease, one was a patient with eosinophilia and one with hepatosplenomegaly; there was also an individual who had been exposed to toxoplasma in the laboratory for many years.

TABLE V CONDITIONS WHICH FAILED TO PRODUCE TOXOPLASMA NEUTRALIZING ANTIBODIES

PATIENT	AGE	DIAGNOSIS
Dho	7 mo	Lamellar demyelinating sclerosis
Aho	18 mo	Lamellar demyelinating sclerosis
Vo	2 yr	Congenital bone abnormalities, mental retardation
But	2½ yr	Encephalitis last 6 weeks (convulsions, ataxia, pasticity, photophobia)
Wal	5 yr	Right cerebral atrophy, epilepsy, convulsion, pasticity
Smi	8 yr	Eosinophilia
Fem	8 yr	Prolonged encephalitis, cortical atrophy, mental retardation
Bal	14 yr	Brain abscess
Dav	15 yr	Acute encephalitis (toxoplasma isolated from animals inoculated with C S F) patient tested seven years later
Fla	18 yr	Hodgkin's disease
Tro	20 yr	Hodgkin's disease
Lap	28 yr	Hodgkin's disease
Ruc	37 yr	Laboratory exposure for many years
Sak	40 yr	Epileptic seizures, abortions
Hes	74 yr	Hepatosplenomegaly, emphysema of gall bladder

Ca. reported by Sabin\*

## DISCUSSION

With the aid of quantitative methods, Sabin and Olitsky<sup>6</sup> were able to demonstrate the development of toxoplasma neutralizing antibodies in experimentally infected monkeys. Later, utilizing the same method, Sabin<sup>5</sup> was able to show the presence of such antibodies in a human patient with toxoplasmosis, and this was soon corroborated by Cowen, Wolf, and Paige.<sup>7</sup> The latter were able to show not only that antibodies were present in the young patients but in some of the mothers as well, thus giving additional evidence for the belief that the disease was congenitally acquired by those patients. Certain discrepancies regarding the presence of neutralizing antibodies were explained when Sabin and Ruchman<sup>8</sup> discovered the extreme lability of the antibody and the ease with which it disappeared from stored serum unless proper precautions were taken for its preservation. Sera kept frozen in dry ice or lyophilized were found to retain their antibody content for long periods of time. With due regard for the instability of the antibody, it was noted that neutralizing substances appeared in the blood stream of monkeys within two weeks after infection and persisted for over a year, which was the longest interval tested. It was also observed that the presence of antibody was not associated with the persistence of parasites in the tissues of the monkeys. Having ascertained that the neutralization test yielded reproducible results, Sabin<sup>1</sup> extended the work to include sera obtained from human beings. It was found that neutralizing antibodies were almost regularly present in infants and children showing psychomotor disturbances with or without hydrocephalus or microcephaly only when these were associated with cerebral calcification or chorioretinitis or both. Corroboration was obtained of the presence of antibodies in the mothers of affected children. Occasionally other members of the immediate family were found to be positive. In a high proportion of patients examined, neutralizing antibodies were found in patients with obscure encephalopathies, in mothers who gave birth to anencephalic, microcephalic, or hydrocephalic infants, and in older children with chorioretinitis as well as some of their mothers. The latter were made the basis of a separate report by Vail and co-workers.<sup>9</sup> Callahan<sup>10</sup> studied the sera of one hundred apparently well adults and found that only 2 per cent were able to neutralize the effects of toxoplasma. Heidelman<sup>11</sup> corroborated the finding of a high incidence of positive sera in patients with congenital chorioretinitis as well as in many of their mothers. The incidence was approximately 10 per cent in cases of acquired chorioretinitis and much less in patients with acquired anterior uveitis. A case diagnosed clinically as toxoplasmic in origin was used by Adams and associates<sup>12</sup> to study the sera obtained from members of the immediate family. They were able to show the presence of antibodies in the patient, the mother and nine of ten siblings. Finally, Johnson<sup>13</sup> corroborated the finding of a high incidence of positive sera among patients with active or inactive central chorioretinitis, especially when cerebral calcification was also present. Among the sera of twenty-eight patients (15 to 50 years of age) with macular chorioretinitis alone, a total of 16, or 57 per cent, was positive. The sera from four individuals (3 to 5 years of age at first examination) who had cerebral calcification in addition to the chorioretinitis were positive. Again the

blood of the mothers (two of two tested) and of some of the siblings (eight of ten tested) of such patients was positive

The results reported in this series of toxoplasma neutralization tests corroborate the general finding of positive sera among patients with hydrocephalus or microcephaly and psychomotor disturbances when these are associated with chorioretinitis, particularly in the macular region of the eye, and with cerebral calcification. The high incidence of positive antibody tests in the mothers of these patients substantiates the evidence that the infection was acquired in utero. In at least one instance however the blood of a child with encephalitis was positive while the mother's blood was negative, indicating that the infection was probably acquired postnatally. It is not known to what extent the presence of antibodies means past infection. Indeed rough estimates place the incidence of neutralization from 2 per cent to about 10 per cent in different regions.<sup>1-10-11</sup> Of greater importance is the constant association of a certain syndrome with the presence of neutralizing antibodies. In this case the problem becomes one of statistical study. Such studies have already demonstrated the close association between positive sera in young children presenting the cardinal signs of congenital toxoplasmosis on the one hand and the presence of antibodies in the mothers' blood on the other. Occasionally other members of the immediate family have had circulating antibodies against toxoplasma giving credence to the belief that the infant acquired the infection in utero at a time when the infection was widespread among some members of the family.

Although no exact figures are available it would appear that antibodies once attained as a result of specific infection persist for long periods of time perhaps indefinitely. The longest period of examination was until about five years after diagnosis at which time the serum neutralized to the same extent as before. Numerous lesser intervals have revealed that the antibodies were still present in the blood stream. For example among the ninety tests performed on seventy two individuals, some repeat examinations with freshly drawn serum samples were included. The specimens were obtained anywhere from two weeks to many months after the initial bleeding tests were performed. Among the twenty two individuals with positive sera nine were retested of which eight remained positive and one became equivocal. Among the fifty negatives five were retested and all remained negative. Seven equivocal individuals were found and of these, four were reexamined at a later date. One remained doubtful two became negative and one turned positive. This last occurred in an infant suspected of toxoplasmosis whose test was equivocal at one month of age but became strongly positive at three months of age. This observation has been pointed out by Sabin<sup>4</sup> and to a certain extent explains the negative and equivocal results obtained by others in an occasional infant with toxoplasmosis. It does not explain the observation by Heidelman<sup>11</sup> of individuals who neutralized and subsequently lost their antibodies. A suggested explanation is that the sera originally were actually equivocal, since we have recorded only one instance in which a positive individual eventually became negative. Even this individual was originally on the borderline of positive and later became

equivocal We thus far have not seen a strongly positive serum that later became completely negative

Negative tests were obtained in many of the disease conditions and the small selection reported here emphasizes the need of tests on additional diseases of obscure etiology to rule out the possibility of toxoplasmosis Such tests<sup>1</sup> have already excluded the possibility of toxoplasmic infection in patients with hydrocephalus, microcephaly, psychomotor retardation, or convulsions not associated with cerebral calcification or chorioretinitis or both All of the sera from ten patients with hydrocephalus or microcephaly in the absence of cerebral calcification or chorioretinitis were negative for neutralizing antibody These, together with the sera from eight of nine patients reported here, make a total of eighteen of nineteen patients tested that failed to show antibodies when neither cerebral calcification nor chorioretinitis was present

#### SUMMARY

The toxoplasma neutralization test performed in rabbits was used to determine the presence of antibodies in various disease conditions Of the sera obtained from seventy-two selected individuals, twenty were positive, forty-eight were negative, and four were equivocal Corroboration was obtained of the high incidence of the positive sera among children showing signs of congenitally acquired toxoplasmosis, namely, convulsions or other signs of central nervous system involvement and hydrocephalus or microcephalus when these were associated with cerebral calcification or chorioretinitis or both Antibodies were regularly present in the blood of the mothers of such patients Antibodies were also found in a child with prolonged encephalitis and in two adults with mental disturbances The toxoplasma neutralizing antibodies persist in the blood stream for at least five years which was the longest interval tested Many conditions of obscure etiology failed to show the presence of toxoplasma antibodies

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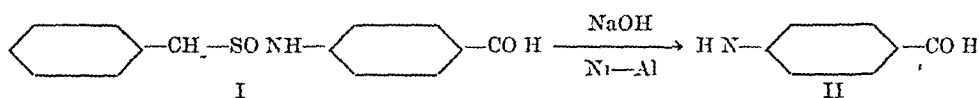
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# LABORATORY METHODS

## A COLORIMETRIC DETERMINATION OF CARONAMIDE

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THE evaluation of a large number of substances of various chemical types for their property of inhibiting the renal elimination of penicillin has led to the selection of 4'-carboxy-phenylmethanesulfonamide (caronamide) for detailed study<sup>1, 2</sup> The extensive pharmacological and clinical evaluation of such an agent requires a method for the quantitative determination of the substance in tissues and in body fluids The convenience and acceptability of the colorimetric method for the quantitative determination of sulfanilamide derivatives<sup>3, 4</sup> led to attempts to adapt this method for the determination of caronamide Since caronamide (I) is a derivative of p-aminobenzoic acid, a cleavage to yield p-aminobenzoic acid (II) would permit the application of the diazotization method to this compound This general colorimetric procedure has been employed for the determination of p aminobenzoic acid and its derivatives in biologic material<sup>5, 6</sup>



Caronamide proved to be resistant to hydrolytic cleavage except under diastic conditions that resulted in the destruction of the p aminobenzoic acid also However, it was found that the action of a powdered nickel-aluminum alloy (Raney catalyst alloy\*) in alkaline solution gave complete and smooth cleavage In this method the reaction of the aluminum in the alloy with the sodium hydroxide results in a vigorous evolution of hydrogen The hydrogen, in contact with the nickel from the alloy, causes hydrogenolysis of the caronamide with the liberation of p aminobenzoic acid which then may be determined by the established procedures

A description of the procedures for the determination of caronamide in urine, in plasma, and in blood is presented in this communication The application of this method to other compounds will be reported later

In the procedures for plasma and blood, 90 per cent alcohol proved to be a satisfactory protein precipitant Acid precipitants such as trichloroacetic acid or p-toluenesulfonic acid were not satisfactory because of the low solubility of caronamide in acid media and because of the large volume obtained when the

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acid filtrate was neutralized and then made sufficiently alkaline for treatment with the alloy. In the case of plasma it is not essential that the protein be precipitated. In the alternative procedure the plasma in alkaline solution is treated directly with the alloy.

# REAGENTS

Sodium hydroxide solution, approximately 5 per cent  
 Raney catalyst alloy  
 Hydrochloric acid, approximately 6 N, the concentrated acid diluted with an equal volume of water  
 n-Octyl alcohol, to prevent foaming with alloy treatment  
 Sodium nitrite, 0.2 per cent solution freshly prepared  
 Ammonium sulfamate 2 per cent solution  
 N (1 Naphthyl) ethylenediamine dihydrochloride, 0.1 per cent solution freshly prepared  
 Ethanol, 90 per cent

# PROCEDURES

For the determination of the p-aminobenzoic acid following the alloy treatment, a slight modification of the procedure described by Eckert is used. N (1 Naphthyl) ethylenediamine is employed as the coupling agent. A Klett Summer on photoelectric colorimeter with a No. 54 green filter was used to read color intensities.<sup>5, 6</sup>

**I Determination in Urine**—Two milliliters of the urine sample are diluted accurately to 10 ml with water\*. One milliliter of this solution is added to 10 ml of 5 per cent sodium hydroxide solution in a 125 ml flask or a large test tube. Two drops of n-octyl alcohol are added and then 0.5 Gm of the Raney catalyst alloy†. The vessel is swirled gently to mix the contents. When the vigorous reaction has subsided, the mixture is heated on a steam bath or in a boiling water bath for twenty minutes. The mixture is cooled and the residual nickel allowed to settle. The aqueous portion is decanted from the nickel and the nickel is washed by decantation four times with distilled water. The aqueous solution and the washings are combined and diluted to 100 milliliters. Instead of washing by decantation the residual alloy may be separated by filtration or centrifugation‡ and the solution made up to 100 milliliters. Ten milliliters of the alkaline solution are added to 4 ml of 6 N hydrochloric acid§ with constant agitation and the acid solution is diluted to 50 milliliters.

To 10 ml of the acidified solution 1 ml of 0.2 per cent sodium nitrite solution is added. The solution is mixed well and allowed to stand for five minutes. One milliliter of 2 per cent ammonium sulfamate solution is added, the solution is mixed and allowed to stand for three minutes. Finally, 1 ml of the 0.1 per cent solution of N (1 naphthyl) ethylenediamine dihydrochloride is added and the solution is mixed. After thirty minutes the intensity of the color is read. Distilled water is used as a blank. The concentration of caronamide in the original urine sample is obtained by reference to a standard curve constructed by plotting colorimeter readings obtained when a series of standard solutions of drug is carried through the foregoing procedure.

**II Determination in Plasma**—A 2 ml sample of plasma is diluted accurately to 20 ml with 90 per cent alcohol and the suspension is thoroughly mixed. After standing for

\*This primary dilution is desirable to minimize foaming during the alloy treatment and to reduce the concentration of caronamide.

†The alloy may be measured conveniently in a small measuring spoon that is shaped to hold approximately 0.5 gram.

‡Caution. The residual nickel must not be allowed to dry, since it is pyrophoric. It should be washed down the drain immediately with a large quantity of water.

§The alkaline solution must be added to the acid. If the addition is in the reverse order a precipitate forms which dissolves rapidly in the excess acid only when the mixture is warmed.



ten minutes the mixture is filtered through coarse paper and 10 ml of the filtrate are added to 10 ml of 5 per cent sodium hydroxide solution. Two drops of n-octyl alcohol are added, followed by 0.5 Gm of the Raney alloy\*. When the first vigorous reaction subsides, the mixture is heated on a steam bath or in a boiling water bath for thirty minutes. The alcohol is allowed to escape during this treatment. The mixture then is cooled, diluted to 100 ml, and filtered†. Ten milliliters of the filtrate are added to 1 ml of 6 N hydrochloric acid‡. After the addition of 1 ml of 0.2 per cent sodium nitrite, the solution is mixed well and allowed to stand for five minutes. One milliliter of 2 per cent ammonium sulfate is added. After three minutes, 1 ml of 0.1 per cent N (1-naphthyl) ethylenediamine dihydrochloride solution is added and the color is allowed to develop for thirty minutes. The color intensity is read, using distilled water as the blank.

The concentrations of caronamide are calculated by reference to a standard curve prepared by plotting colorimeter readings obtained by using this procedure on samples of plasma containing known quantities of the drug.

*III Alternative Determination in Plasma*—A 1 ml sample of plasma is diluted with 10 ml of 5 per cent sodium hydroxide solution. To this are added two drops of n-octyl alcohol and 0.5 Gm of Raney alloy. When the first vigorous reaction has subsided, the reaction mixture is heated on the steam bath for thirty minutes. The mixture then is cooled, diluted to 100 ml, and filtered†.

A 10 ml aliquot of the filtrate is added to 1 ml of 6 N hydrochloric acid‡, after which 1 ml of the 0.2 per cent sodium nitrite solution is added. This is mixed well and allowed to stand for five minutes. One milliliter of 2 per cent ammonium sulfate is added. After the solution has stood for three minutes, 1 ml of 0.1 per cent N (1-naphthyl) ethylenediamine dihydrochloride solution is added and the color is allowed to develop for thirty minutes. Intensity of color is read with the colorimeter. In order to correct for a slight turbidity that appears with some plasma specimens, a blank is prepared from a second 10 ml sample of the alkaline filtrate from the alloy treatment by omitting the sodium nitrite.

Concentrations of caronamide are calculated from a standard curve prepared by plotting colorimeter readings obtained by using the foregoing procedure on samples of plasma containing known quantities of drug.

*IV Determination in Blood*—A 2 ml sample of blood is added to 2 ml of water and the mixture is allowed to stand for ten minutes in order to allow hemolysis of the cells. This mixture is diluted accurately to 20 ml with 90 per cent alcohol. The suspension is mixed thoroughly, allowed to stand for ten minutes, and filtered. Ten milliliters of the filtrate are carried through the alloy treatment as described in II. A standard curve is constructed from values obtained with blood to which known amounts of the drug have been added.

*Results With Standard Solutions*—Solutions containing known amounts of p-aminobenzoic acid in water, in urine, in plasma, and in blood were prepared and run through the alloy treatment according to these procedures. The amounts of p-aminobenzoic acid recovered were calculated by reference to a plot of the colorimeter readings that were obtained on standard aqueous solutions not subjected to the alloy treatment. The results, which are tabulated in Table I, show that recoveries generally were 80 to 90 per cent. Therefore, 10 to 20 per cent of the p-aminobenzoic acid is lost during the alloy treatment. However, for each procedure the recoveries are fairly constant and reproducible.

Similarly, solutions containing known amounts of caronamide were subjected to these procedures and the recoveries of p-aminobenzoic acid were cal-

\*See footnote 1 on page 97

†See footnote 2 on page 97

‡See footnote 3 on page 97

TABLE I RECOVERY OF P AMINOBENZOIC ACID

PROCEDURE	PAB ADDED (MG/100 ML)	PAB RECOVERED	
		MG/100 ML	%
<i>In Water</i>			
I	500	424	85
I	200	206	82
I	100	84	84
III	20	17	85
III	10	8.3	83
III	5	4.2	84
<i>In Urine</i>			
I	500	456	91
I	200	228	91
I	100	90	90
<i>In Plasma</i>			
II	20	16.6	83
II	10	8.6	86
II	5	4.3	86
III	20	16	80
III	10	8	80
III	5	4	80
<i>In Blood</i>			
IV	20	15.6	78
IV	10	8.2	82
IV	5	4.1	82

culated. Again the recoveries were generally between 80 and 90 per cent and were constant and reproducible for each procedure. Since the recoveries were essentially the same after the alloy treatment of either p aminobenzoic acid or caronamide it appears that the caronamide is split quantitatively but that an average of 15 per cent of the liberated p aminobenzoic acid either is destroyed during the treatment or is lost, possibly by adsorption on the finely divided nickel.

TABLE II RECOVERY OF P AMINOBENZOIC ACID FROM CARONAMIDE

PROCEDURE	CAPRONAMIDE ADDED (MG /100 ML )	PAB EQUIVALENT (MC /100 ML )	PAB RECOVERED	
			(MG /100 ML )	%
In Water				
I	1000	470	405	86
I	500	235	202	86
I	200	94	84	89
In Urine				
I	1000	470	408	87
I	500	235	205	87
I	200	94	84	89
In Plasma				
II	40	18.8	16.4	87
II	20	9.4	8.3	88
II	10	4.7	4.1	87
III	40	18.8	14.4	76
III	20	9.4	7.2	76
III	10	4.7	3.2	68
In Blood				
IV	40	18.8	15.4	82
IV	20	9.4	7.8	83
IV	10	4.7	4.0	85

From these data it is evident that standard reference curves for use in routine determinations may be constructed from the colorimeter readings obtained when urine, plasma, or blood containing known amounts of caronamide are run through the appropriate procedure. From Table I and Table II it will be noted that procedure III, which is run on plasma without removal of protein, gives lower recoveries than does procedure II. Nevertheless, this procedure has proved to be useful in the analyses of a large number of routine specimens where extreme accuracy was not required.

For the best results the acid solutions used for the diazotization and coupling in these procedures should correspond to a caronamide concentration of between 0.15 and 0.4 mg per 100 ml, or approximately 0.075 to 0.2 mg per 100 ml of p-aminobenzoic acid. At these concentrations the scale readings of the colorimeter lie between 100 and 300.

With the dilutions employed in these procedures (1:2,500 for urine, and 1:100 for plasma and blood), caronamide concentrations in urine from 200 to 1,000 mg per 100 ml, and in plasma or blood from 5 to 50 mg per 100 ml, can be determined satisfactorily.

#### SUMMARY

A procedure for the colorimetric assay of caronamide is described. Treatment in aqueous alkali with nickel-aluminum alloy quantitatively liberates p-aminobenzoic acid which then is determined by established procedures.

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# A COMPENSATING PLETHYSMOKYMOGRAPH FOR MEASURING BLOOD FLOW IN HUMAN EXTREMITIES\*

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ROCHESTER MINN

ACCURATE measurement of blood flow through the extremities of human beings has proved to be a fairly difficult problem. That plethysmographic methods are not completely satisfactory is attested by the fact that for the past thirty years new improvements of the original plethysmograph invented by Biot<sup>1</sup> and Russell<sup>2</sup> have been presented frequently. The skepticism of Pezzali<sup>3</sup> concerning the value of this method for measuring blood flow has frequently proved to be justified although the plethysmograph has yielded a large volume of valuable physiologic information of qualitative if not quantitative significance. Unfortunately, however, most of the physical and physiologic principles on which the method is based have never been adequately analyzed. In fact, it has been demonstrated in only one organ<sup>3</sup> (the kidney) that blood flow is measured by the plethysmographic method. Landowne and Katz<sup>4</sup> have presented an admirable critique of the plethysmographic method of measuring blood flow in human beings.

## PRINCIPLE OF PLETHYSMOKYMOGRAPHY

The term plethysmography is derived from Greek and literally means "the recording of the curve of filling." An organ or a portion of an extremity is sealed in a leakproof plethysmograph. A sphygmomanometer cuff connected to a pressure reservoir is placed about the extremity of the subject just proximal to the plethysmograph. This cuff is called the collecting cuff and its function is to produce occlusion of the veins. Sudden inflation of the collecting cuff to a pressure below diastolic blood pressure yet above venous pressure, traps incoming arterial blood within the portion of the extremity distal to the collecting cuff and thereby causes engorgement and increase in volume of the portion of the extremity which lies inside the plethysmograph. Connected by tubing to the inside of the plethysmograph is a recording device which is activated by a change of pressure within the plethysmograph. This change of pressure within the plethysmograph and recorder is induced by a change in volume of the enclosed portion of an extremity. The recorder may trace on a smoked drum or reflect a beam of light to record on moving photosensitive paper. The latter method is called the optical system of recording and is the more satisfactory of the two.

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when sensitivity is essential. The rate of increase in volume of the portion of the extremity enclosed in the plethysmograph is measured by the excursion of the recorder and a timing device. The increase in volume per unit of time, so obtained, is assumed to be an indication of actual blood flow to the portion of the extremity enclosed in the plethysmograph. The record obtained is called a "flow curve." Fig 1 shows several types of flow curves which were obtained in this study.

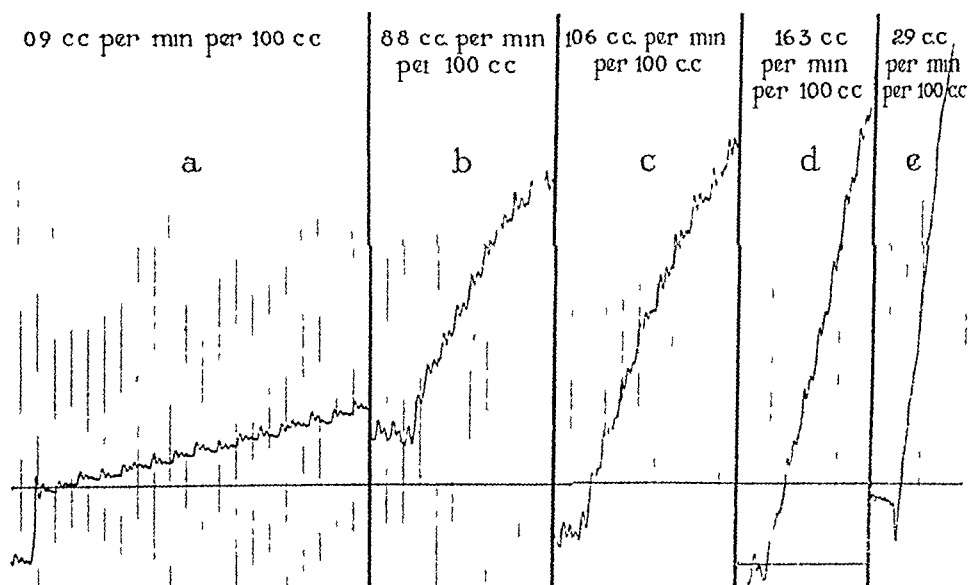


Fig 1—Typical blood flow curves obtained from the arms of human beings by the plethysmographic method. Each record was taken on a different subject. Calculated volume flow for each curve is given at the top of the respective tracing.

The purpose of this communication is to describe a compensating plethysmographic method which we have developed. After careful analysis and appraisal of this and various other methods we have reached the conclusion that this compensating plethysmograph most nearly satisfies the criteria for accurate determination of blood flow in human extremities. The following are the main requirements which we consider essential for the accurate determination of blood flow through human extremities by the plethysmographic method:

- 1 Accurate measurement of pulsating changes in volume per second
- 2 Approximately normal environmental conditions
- 3 Complete and sudden arrest of the return of venous blood from the extremity without obstruction of the flow of arterial blood into the extremity
- 4 Elimination of or compensation for mechanical errors due to
  - (a) Displacement of fluid and tissue from beneath the collecting cuff into the plethysmograph during inflation of the collecting cuff
  - (b) Respiratory and other motions of the extremity
  - (c) Differences in calibration of the recording device due to the differences in the volume of various extremities

- (d) Changes in environmental temperature and pressure
- (e) Leaks in the apparatus
- (f) Slight variations in camera speed

Air was preferred to water as an environmental medium inside the plethysmograph for the following reasons

- 1 Water constitutes an abnormal environmental medium
- 2 The inertia of water might tend to slow down rapid changes in volume of an extremity
- 3 External water pressure may prevent increases in volume of the venous system
- 4 We desired a method of plethysmography which would be applicable to the leg and foot as well as to the arm and hand. With water as an environmental medium, the patient's knee must be at a higher level than the foot in order to prevent leakage of water about the point where the plethysmograph is sealed to the patient's extremity. If the patient's foot is at a lower level than his knee venous pressure and the external pressure of the surrounding water are greater in the foot than in the leg and arterial blood should flow into the leg more easily than into the foot.

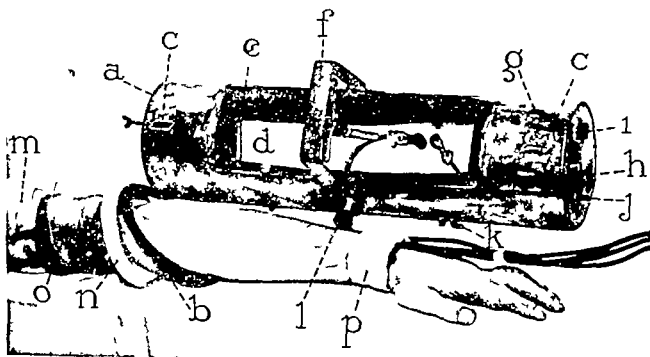


Fig. —The arm plethysmograph with the arm prepared for insertion into the plethysmograph. *a* Open end of the plethysmograph. *b* Braided ring for clamping the occluding diaphragm to the open end of the plethysmograph (this is passed over the diaphragm before clamping). *c* Metal straps for swinging the plethysmograph from above. *d* Plate glass window resting on rubber gasket. *e* Rubber gasket. *f* Ring clamp holding the window in place. *g* Inlet for callibrator. *h* Outlet for a Marey tambour. *i* Outlet for wide bore tubing leading to the compensating spirometer recorder. *j* Outlet to the recorder for a finger plethysmograph. *k* Inlet as the inlet for perfusing the plethysmograph when calibrating. *l* Inlet for inflation of the wrist cuff. *m* Inlet for four electric thermocouples. *n* Rubber sleeve. *o* Rubber leeve everted over a sponge rubber cellulose diaphragm. *p* Collecting cuff. *q* Wrist cuff.

#### DESCRIPTION OF APPARATUS\*

*Plethysmograph (Fig. 2)*—A copper cylinder 18 inches (45.7 cm) long and 6 inches (15.2 cm) in diameter is closed at one end and open at the other. In the side of the cylinder is a rectangular opening 4 by 8 inches (10.2 by 20.3 cm) in diameter. A plate glass window held in place by a strong spring clamp

\* We are indebted to Mr. A. N. Fort for his technical assistance.

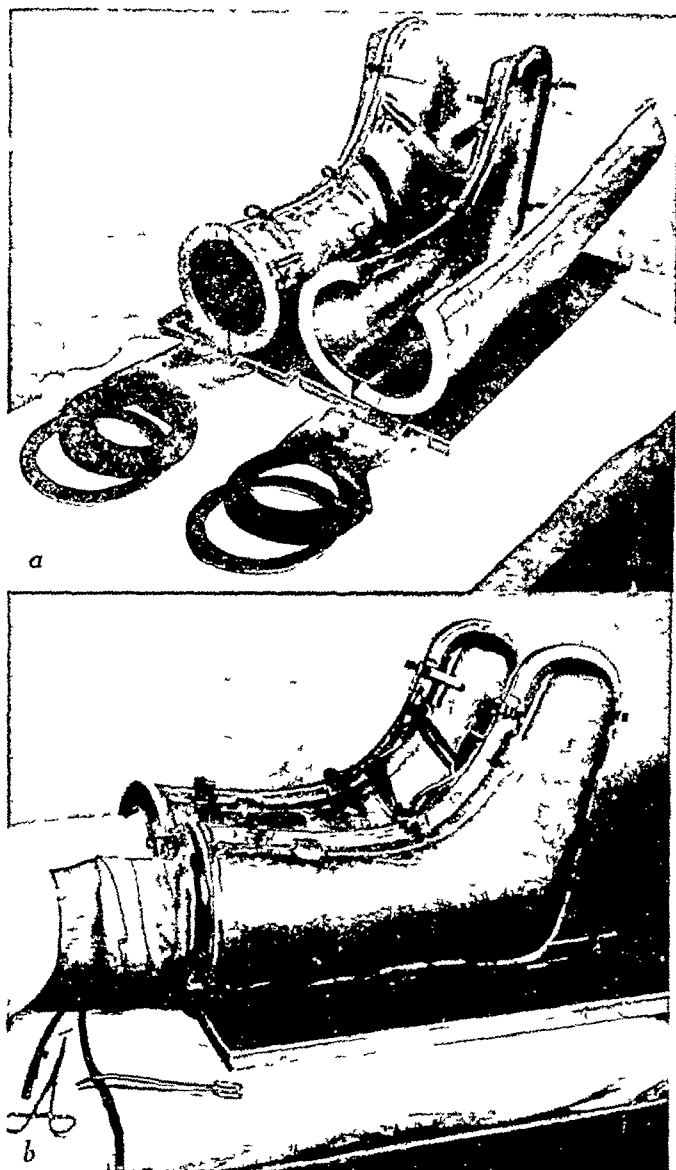


Fig 3—Leg plethysmograph showing the various stages in the preparation of the leg for making an airtight seal around the leg when inserted into the plethysmograph without interfering with the circulation. The various outlets corresponding to those of the arm plethysmograph are located on the medial surfaces. *a* Constituent parts of leg plethysmograph. *b* plethysmograph applied to legs and made airtight without interference with circulation.

rests on a sponge rubber gasket which occludes the juncture between the edge of the rectangular window and the plate glass. When the plethysmograph is horizontal, the window is on the upper side and permits visualization of the limb. On the right side of the plethysmograph is a round opening large enough to convey the lead wires of electric thermocouples for determination of skin temperatures. Outlets for a Marey tambour and a calibrator are placed at the top of the distal end of the plethysmograph. On the side of the plethysmograph is a

connection for inflating a wrist cuff. On the distal end is an outlet for a recorder connected to a finger plethysmograph and another outlet for a recorder connected to the arm plethysmograph. The instrument is suspended and swung by means of a rope from a point above and moves freely from side to side or back and forth with respiratory and other involuntary motions of the extremities. The plethysmograph is rendered airtight by means of a thick, stiff sponge rubber diaphragm called the occluding diaphragm which is clamped over the open end of the plethysmograph by a heavy brass ring.

Plethysmographs for the lower extremities were made of cast aluminum as shown in Fig. 3. The major principle of their operation is the same as for the arm plethysmographs described previously. The plethysmographs were painted flat black in order to facilitate heat transfer and prevent abnormal variations of temperature.

*Blank Plethysmograph*—The blank plethysmograph is a replica of the above mentioned plethysmograph and is used in conjunction with compensating spirometers (see next section) to eliminate the errors induced in the estimation of blood flow by changes in the environmental temperature and pressure.

*Compensating Spirometers (Fig. 4)*—The recording device which we have named a compensating spirometer, is made up of two spirometers identical in capacity and form. Each spirometer is made of thin shim brass in the shape of a hollow, truncated wedge. One of the broad sides of the wedge is omitted thus forming an inverted cup when the wedge is placed with the open side down. Each spirometer rotates about an axle mounted on ball bearings. The axles of the two spirometers lie in the same axis. Each spirometer floats on kerosene or "finol" contained in a square metal pan. A metal pipe pierces the bottom of the pan passes up through the contained kerosene and opens into the air pocket in the spirometer. Wide bore rubber or plastic tubing connects one spirometer to the plethysmograph and the other to the blank plethysmograph. In this way the enclosed space between the top of each spirometer and the surface of the kerosene is in communication with the respective plethysmograph. As the intraplethysmographic volume increases the spirometer rises as the volume decreases it falls. The spirometer pans are mounted side by side and 1 cm apart. An extension of the axle of each spirometer projects into the space between them and to each extension a small mirror is cemented.

The two spirometers are so placed that a single filament lamp throws a horizontal beam of light through the space separating the spirometer pans and into the camera. The mirror mounted on the axle of the spirometer connected to the blank plethysmograph reflects the beam of light from the lamp backward and onto the mirror attached to the spirometer connected to the true plethysmograph. The latter mirror reflects the beam of light forward through a focusing lens into the camera. Thus the optical system is so arranged that an increase in volume of the blank plethysmograph causes a downward deflection of the recording beam while an increase in volume of the true plethysmograph causes an upward deflection of the light beam. Any factor which changes the volume of both the true plethysmograph and the blank plethysmograph simultaneously to the same degree and in the same direction does not appreciably



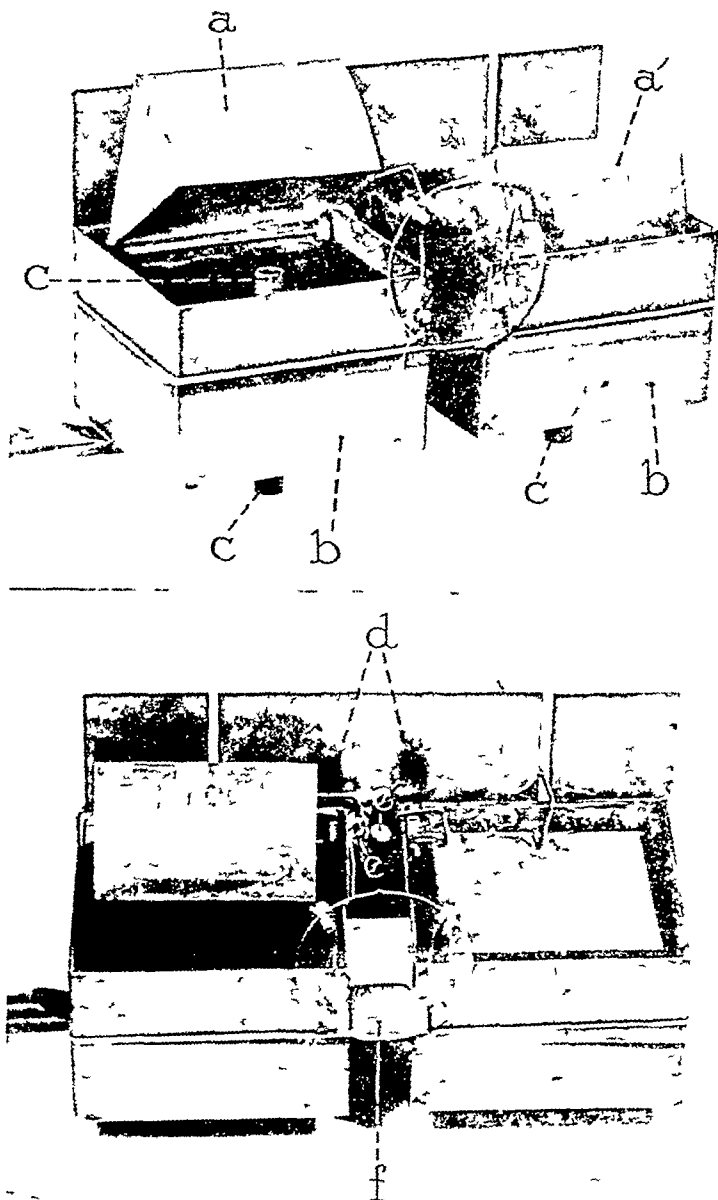


Fig 4—Compensating spirometer recorder *a* Spirometer for recording volume change in the blank plethysmograph *a'* spirometer for recording volume changes in the true plethysmograph *b* metal pans containing fluid which seals the spirometers *c* metal pipes piercing the bottoms of the pans for connection with the plethysmographs *d* extension of the axes of the spirometers into the space between the pans *e* mirror facing backward *e* mirror facing forward *f* focusing lens

alter the position of the recording beam. Since changes in room temperature and pressure affect the true plethysmograph and the blank plethysmograph equally and simultaneously, the errors caused by these changes are eliminated from the flow curve (Fig 5)

The mechanism for compensation is also demonstrated in Fig 6. If venous occlusion is made in both extremities simultaneously, the deflection of the beam

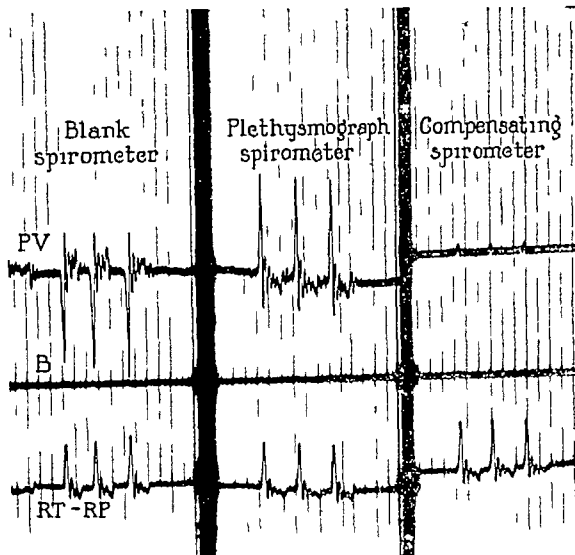


Fig. 5—Tracings illustrating the mechanism by means of which compensating spirometers compensate for fluctuations in room temperature and pressure. PV, Plethysmograph volume; B, base line; RT-RP, room temperature and room atmospheric pressure.

Fluctuations in atmospheric pressure were induced by suddenly opening and closing the door of the laboratory three times during each experiment. Such fluctuations are recorded in the RT-RP line. Note that they are about equal in each of the three experiments.

Blank spirometer. Tube leading to the plethysmograph was clamped so that only the spirometer recording volume changes in the blank plethysmograph recorder. Note that the major deflection of the spirometer is downward.

Plethysmograph spirometer. Tube leading to the blank plethysmograph spirometer was clamped so that only the plethysmograph spirometer recorder. The major deflection is now upward.

Compensating spirometer. Both the blank and plethysmograph spirometers recorded. The downward deflection of the former is neutralized by the upward deflection of the latter and no significant deflection of the recording beam occurs despite changes in atmospheric pressure.

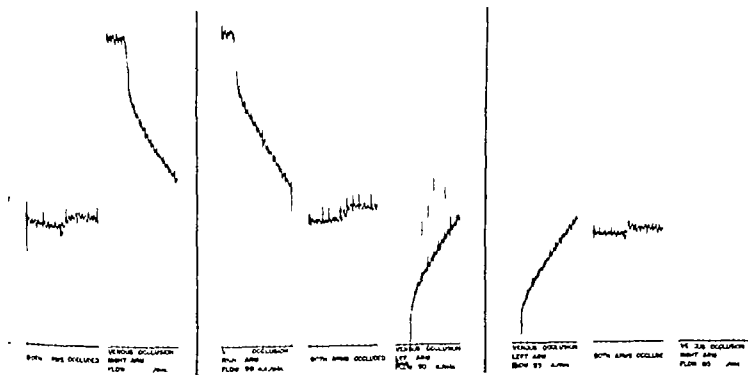


Fig. 6—Tracings illustrating the mechanism of compensation by use of compensating spirometer recorder. Blood flow curves were taken from the left and right forearm of the same individual. Simultaneous venous occlusion of both arms caused only an insignificant deflection of the beam.

is insignificant, yet venous occlusion of the left or right limb alone will give a typical flow curve for that limb. It is clearly demonstrated that the deflection of the light beam by occlusion of the right arm is downward and its base line had to be made at the top of the camera slit. Venous occlusion of the left arm, however, deflects the beam in a direction diametrically opposite to that of the right and its base line, therefore, is made at the bottom of the camera slit. Both flow curves for the right and left forearms can be made separately and calculated as shown in Fig. 6. Simultaneous occlusion of both arms produces practically identical changes in volume in both arms at the same time and therefore the upward deflection of the beam produced by one arm annuls the downward deflection produced by the other. Hence, by the use of compensating spirometers simultaneous changes in the limbs similar in magnitude and direction are taken care of without significant error.

*Marcy Tambour* —A Marcy tambour with an optical system of recording is used to test for the presence of leaks. To test for leaks, the tube leading to the compensating spirometer from the plethysmograph is clamped. Enough air is injected into the plethysmograph to induce a pressure of 15 cm. of water and the deflection of the beam of light reflected from the rubber tambour is noted. A persistent fall in pressure is indicative of a leak in the apparatus.

#### PROCEDURE FOR MAKING AN AIRTIGHT SEAL BETWEEN THE LIMB AND THE PLETHYSMOGRAPH WITHOUT INTERFERING WITH THE CIRCULATION

The procedure that was used for making an airtight seal consisted of coating the part of the limb proximal to the plethysmograph with surgical jelly before inserting it into a thin rubber sleeve 8 to 10 inches (20.3 to 25.4 cm.) long. The sleeve fits loosely around the limb (Fig. 2). A disk of sponge rubber  $\frac{1}{2}$  inch (1.3 cm.) thick and with a diameter equal to that of the inlet of the plethysmograph had a hole cut into it conforming to the contour of the limb but with a slightly greater circumference. The limb is slipped through the hole in the disk and the distal end of the rubber sleeve is everted over the rim of the disk. A bandage is wrapped snugly but lightly over the rubber sleeve and limb proximal to the disk. This helps to support the disk and sleeve, insure airtightness, and eliminate the presence of pockets under the sleeve. The distal surface of the rubber disk is coated with surgical jelly and clamped to the rim of the plethysmograph after insertion of the limb into proper position in the plethysmograph. The whole limb is then elevated sufficiently to insure venous drainage. An ordinary sphygmomanometer cuff used for children is wrapped around the limb about  $\frac{1}{2}$  inch (1.3 cm.) proximal to the occluding disk and is used as a collecting cuff.

#### PHYSICAL CHARACTERISTICS OF THE APPARATUS

*Comparison of Various Types of Recorders* —In general, the various types of recorder used by previous investigators fall into two groups:

1. Those which maintain their deflection to a given increase of intra-plethysmographic volume by means of pressure built up within the plethys-

mograph by the increment of volume. The Marey tambour and glass spoon manometer are representative of this group. This group hereafter will be called pressure recorders.

2 Those which will tend to maintain their deflection without pressure though a slight change in intraplethysmographic pressure is necessary to cause the initial deflection. Biot's bellows, piston recorders and spirometers fall into this group. A single spirometer and compensating spirometers have been selected for study. This group hereafter will be called volume recorders.

The size of the tubing connecting the recorder to the plethysmograph should vary with the type of recorder. With a pure pressure recorder such as the glass spoon manometer, small bore pressure tubing yields the best results. With volume recorders such as the compensating spirometers, large bore tubing is essential. We have found tubing with an internal diameter of 1.5 cm. to be most satisfactory. Tubing of 1.00 cm. internal diameter or less introduces a definite lag in the recorder. Biot's and Abramson Zizeka, and Marius<sup>8</sup> also found wide bore tubing essential when using volume recorders.

*Frequency of the Recorders*—A Dile Schuster pump was so arranged that 5 cc. of air were injected into and extracted from the plethysmograph at the rate of about 50 cycles per second. The compensating spirometers failed to register these oscillations. The speed of the pump was then gradually decreased and it was found that the compensating spirometers responded to the alternate suction and pressure when these cycles occurred about twelve times per second. The pump was further slowed until the excursion of the compensating spirometers was maximal to each cycle. This occurred at 3.3 cycles per second and was taken to represent the natural frequency of the recorder. By the same method the natural frequency of a representative Marey tambour was 10 cycles per second, of a glass spoon manometer 20 cycles per second, and of a single one of the compensating spirometers 3.3 cycles per second.

*The Effect on Various Recorders of Different Volumes of Air in the Plethysmograph*—The rubber tambour and glass spoon manometers gave increasingly higher deflections for the same increment of plethysmographic volume as the arm volumes increased. Single spirometers and the compensating spirometer gave practically identical deflections no matter what the intraplethysmographic volume of air might be.

When the volume of air in the plethysmograph compensating spirometer unit was changed from 9.950 to 9.960 cc., a deflection of 9 cm. occurred. When the volume was changed from 52 to 62 cc., a deflection of 9.15 cm. occurred (Fig. 7).

Hence, if one uses a pressure recorder an individual calibration chart must be obtained for each subject investigated since arm volume varies with each individual. On the other hand, with a volume type of recorder one may use the same calibration chart for any subject.

*The Effect of Various Recorders on Pressure Within the Plethysmograph*—With a typical Marey tambour, slow injection of 10 cc. of water into the plethysmograph induced an intraplethysmographic pressure of about 90 mm. of water.

Slightly greater pressures were induced by the same amount of water injected into the plethysmograph when a glass spoon manometer was used for recording. With the compensating spirometer a change of 0.5 mm of water pressure will move the recorder appreciably. When properly balanced the compensating spirometer maintains a given deflection without appreciable change of pressure.

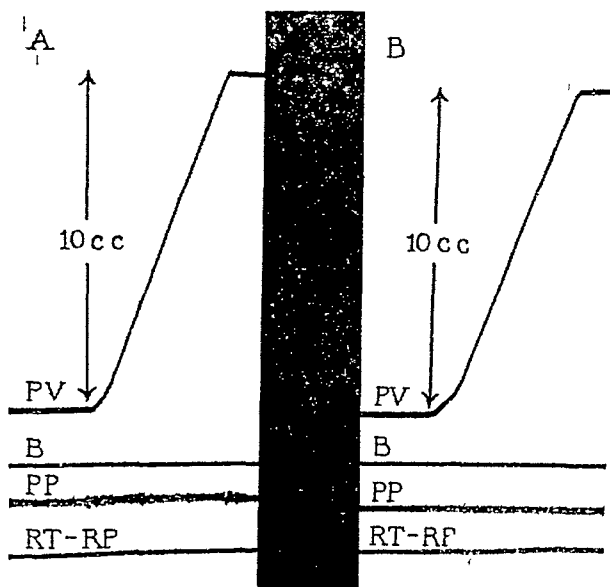


Fig. 7—The effect of varying plethysmographic volume on the deflection of compensating spirometers. PV, Plethysmograph volume; B, base line; PP, pressure in the plethysmograph recorded by a Marey tambour; RT-RP, room temperature and room atmospheric pressure.

A, Volume of the plethysmograph-compensating spirometers unit changed from 9.950 cc to 9.980 cc by the injection of 10 cc of air into the plethysmograph. The deflection of the recorder was 9.0 centimeters.

B, Plethysmograph excluded from the plethysmograph-compensating spirometers unit leaving a volume of 52 cubic centimeters. The injection of 10 cc of air into the compensating spirometers induced a deflection of 9.15 centimeters.

*The Effect on Various Recorders of Changing Environmental Pressure and Temperature*—Ellis<sup>9</sup> in 1885, indicated that an air plethysmograph is very sensitive to changes of temperature. Turner,<sup>10</sup> in 1937, re-emphasized this point. We have found that all four types of recorders studied were very sensitive not only to changes in environmental temperature but also to air pressure. Single spirometers and Marey tambours were particularly vulnerable to errors induced by changes in environmental pressure or temperature. In fact it was impossible to obtain accurate flow curves on a windy day or in a place where doors were continually being opened and closed. On the other hand, the compensating spirometers eliminate errors induced by changes of room temperature or pressure, as discussed in describing the recorder (Fig. 5).

#### CALIBRATION OF THE PLETHYSMOGRAPH, USING THE COMPENSATING SPIROMETER FOR RECORDING

We thought it of interest to compare calibrations of the plethysmograph by the usual method of injecting known volumes of fluid into the plethysmograph and by a method whereby the plethysmograph is perfused with known

pulsating flows By means of the Dale Schuster pump one can deliver very nearly constant pulsating flows of amounts of fluid varying from 0 to 500 cc per minute The pulse contour of the instrument simulates that of the heart We have been unable to find any reference to the calibration of a plethysmograph by the use of known pulsating flows

#### *Method —*

1 *Injecting Known Volumes of Fluid Into the Plethysmograph by Means of a Syringe or Burette* A section of rubber Penrose tubing was placed in the plethysmograph, extending through its entire length The tube was clamped at its distal end The other end opened to an outlet in the proximal end of the plethysmograph where it was connected to the delivery end of a certified 100 cc burette, accurate to 0.05 cc, and filled with water Increments of volume injected from the burette were then plotted against the actual deflections of the recording beam (Fig 8, line B) Rapid injection from a syringe or slow injection from the burette gave the same calibration curve

2 *Perfusing the Plethysmograph With Known Pulsating Flows* The Penrose tubing was arranged in the same way inside the plethysmograph except that the end which had been clamped was fastened to an inlet through the distal end of the plethysmograph A stopcock was inserted in the outlet of the Penrose tubing Water was perfused through the tubing by means of a Dale Schuster pump The perfused water was measured in cubic centimeters per minute at least twice before and once after each flow curve was taken The stopcock in the outlet was suddenly closed, thus trapping water within the Penrose tubing, and a tracing of the increase in intraplethysmographic volume per unit of time was obtained (Fig 9) Flow curves of perfused flows ranging between 10 and 400 cc per minute were then obtained and the slope of each flow curve was drawn Increments of volume of water collected were then plotted against actual deflections of the recording beam (Fig 8, lines A, C, and D)

#### *Method of Measuring Unknown Flows Produced by a Mechanical Schema —*

Having obtained a calibration curve one can deduce accurately the increase in volume within the plethysmograph from the resultant excursion of the recording beam Unknown flows produced by a mechanical schema are measured as follows The plethysmograph is perfused with an unknown pulsating flow delivered by a Dale Schuster pump as described previously under Calibration of the Plethysmograph The slopes of the flow curves obtained when compensating spirometers are used for recording are practically straight lines and therefore the flow may be calculated by any one of the following methods (1) slope of the flow curve, (2) actual increment of volume per pulse beat, or (3) actual deflection of the recorder per unit of time All methods give identical results within the experimental error of about 1 per cent We have used calculations derived from the slope of the flow curve since this was found to be the most convenient method The slope of the flow curve is drawn A three, six or ten second interval of time is marked off along the base line of the flow curve from the point where the slope line intersects the base line From the point so obtained, a perpendicular is erected to intersect the slope line The length of

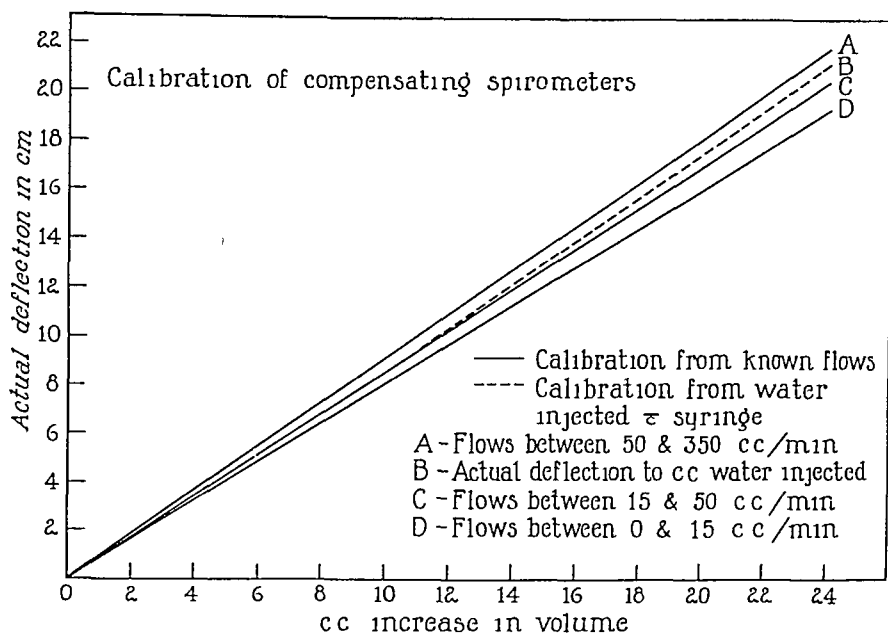


Fig 8—Calibration of compensating spirometers with flows of various magnitudes

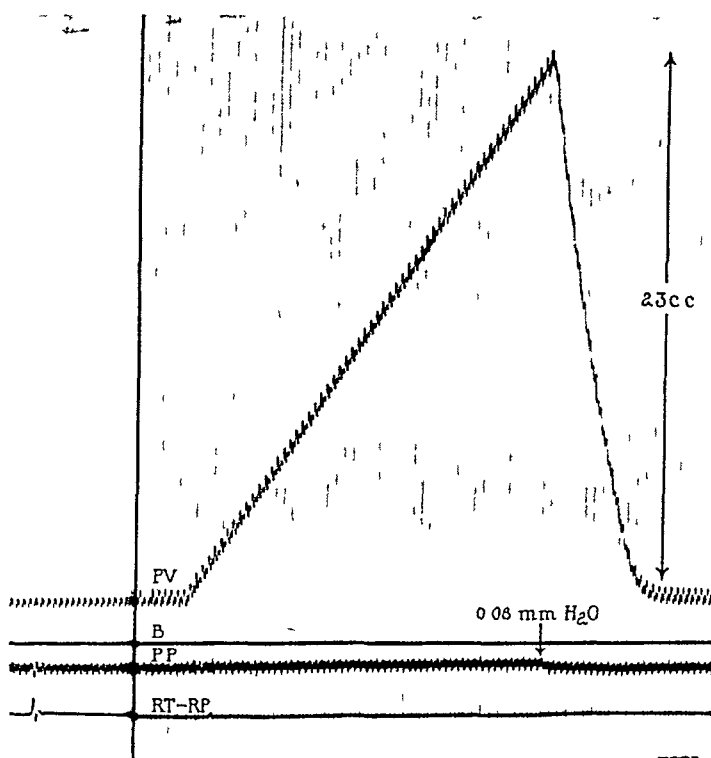


Fig 9—Typical flow curve obtained by perfusing the plethysmograph with known pulsating flows and using compensating spirometers for recording PV, Plethysmographic volume recorded by compensating spirometers B base line PP plethysmographic pressure recorded by a Marey tambour (note that the maximal sustained pressure in the plethysmograph is 0.05 mm of water) RT-RP, room temperature and room atmospheric pressure

this perpendicular line represents the amount of air displaced from the plethysmograph into the recorder during the interval of time used. From the calibration curve, the length of the fore mentioned perpendicular line may be easily converted to cubic centimeters of volume added to the plethysmograph per unit of time and this figure then may be expressed as cubic centimeters of flow per minute.

*Experimental Error of the Plethysmograph When a Mechanical Schema Was Used for Perfusion and the Compensating Spirometer Was Used for Recording*—Hewlett and Van Zwaluwenburg,<sup>11</sup> in 1909 estimated that their apparatus came within 20 per cent of measuring true blood flow in favorable cases. On the other hand, Stead and Kunkel<sup>12</sup> in 1938, estimated the instru-

TABLE I COMPARISON OF ACTUAL FLOW AND CALCULATED FLOW IN A MECHANICAL SCHEMA USING COMPENSATING SPIROMETERS FOR RECORDING

EXPERIMENT	ACTUAL FLOW (CC PER MIN.)	CALCULATED FLOW (CC PER MIN.)	PERCENTAGE ERROR OF CALCULATED FLOW
1	11.3	11.5	+1.8
2	12.5	12.5	0
3	12.5	12.5	0
4	12.5	12.5	0
5	13.0	13.0	0
6	17.0	17.0	0
7	17.5	17.0	-2.8
8	17.5	17.5	0
9	17.5	17.5	0
10	17.5	17.0	-2.8
11	17.7	18.0	+1.7
12	21.3	21.5	+0.9
13	45.0	40.5	-10.0*
14	68.0	68.0	0
15	68.0	68.0	0
16	70.5	69.0	-2.1
17	100.2	100.0	-0.2
18	116.5	119.0	+2.1
19	116.5	118.0	+1.3
20	117.5	119.0	+1.3
21	122.0	120.0	-1.6
22	140.0	138.0	-1.4
23	146.0	142.0	-2.7
24	146.0	145.0	-0.7
25	152.0	151.0	-0.6
26	155.0	150.0	-3.2
27	160.0	161.5	+0.9
28	160.0	159.0	-0.6
29	160.0	160.0	0
30	224.0	221.0	-1.3
31	224.0	220.0	-1.8
32	224.0	223.0	-0.4
33	224.0	220.0	-1.8
34	224.0	221.0	-1.3
35	282.0	278.5	-1.2
36	282.0	276.0	-2.1
37	282.0	279.5	-0.9
38	282.0	275.0	-2.5
39	340.0	339.0	-0.3
40	340.0	329.0	-3.2
41	340.0	337.5	-0.7
42	340.0	343.5	+1.0
43	340.0	339.0	-0.3
Average error			0.58%

No explanation could be found to account for the magnitude of this error.



mental error of their plethysmograph to be  $\pm 0.1 - 3$  per cent. However, they found it necessary to add 13 per cent to the flows calculated from calibration curves obtained by the injection of air into the plethysmograph from a syringe. Primzmetal and Wilson,<sup>17</sup> using an adaptation of Lewis and Grant's<sup>14</sup> instrument (1925), estimated the experimental error of the apparatus to be 15 per cent. Killian and Oelassen<sup>12</sup> estimated the experimental error of a modification of Hewlett and Van Zwaluwenburg's apparatus to be  $\pm 15$  per cent.

The compensating spirometer recorder was studied in detail concerning its accuracy in measuring flows within the usual limits of flow encountered in the human arm. The findings are shown in Table I. The person who calculated the flow from flow curves had no idea whatsoever as to what the actual flows were. The results may be summarized as follows. When compensating spirometers are used for recording pulsating flows produced by a mechanical schema, 98 per cent of the calculated flows lie between  $\pm 2$  and  $-4$  per cent of the actual flow. 95 per cent of the calculated flows lie between errors amounting to  $\pm 2$  and  $-3.5$  per cent, while 82 per cent of flows lie between  $\pm 2$  and  $-2$  per cent of the actual flow. The average error in calculating flows was  $-0.88$  per cent.

#### ANIMAL EXPERIMENTS DESIGNED TO TEST THE VALIDITY OF THE FUNDAMENTAL PRINCIPLES OF PLETHYSMOGRAPHY

*Does the Plethysmograph Measure Blood Flow?*—The increase in volume of a limb after inflation of a collecting cuff might be due to trapping of incoming blood within the limb, displacement of fluid and tissue distally from beneath the collecting cuff, or transudation of fluid from the capillaries to the tissues of the limb. In the estimation of blood flow by the plethysmographic method it is assumed that the trapping of incoming blood is the chief cause of the increase in volume of the limb. The initial portion of the flow curve represents displacement of fluid and tissue from beneath the collecting cuff and is disregarded in estimating blood flow as was discussed by Hewlett and Van Zwaluwenburg,<sup>16</sup> or is eliminated from the flow curve mechanically, as discussed by Wright and Phelps,<sup>17</sup> or manually, as discussed by Hewlett and Van Zwaluwenburg.<sup>18</sup> Davis and Jones<sup>19</sup> found that, after occlusion of the return of venous blood from the legs, edema formed in the leg at the rate of 0.017 cc per minute per 100 cc of tissue when the environmental temperature was 16° C. At 42° C 0.07 cc of edema fluid formed each minute per 100 cc of leg tissue. If one applied the latter findings to a human arm of 1,500 cc volume even in a hot environment, only about 0.18 cc increase in volume would occur during ten seconds after inflation of a collecting cuff. Krogh, Landis and Turner<sup>20</sup> found that fluid accumulated in tissue spaces when venous pressure exceeded 15 to 20 cm of water. When venous pressure exceeded 17 cm of water, the rate of filtration was directly proportional to the increase in venous pressure and an increase in venous pressure of 1 cm of water increased filtration rate by 0.0023 cc per minute per 100 cc of arm. For an arm of 1,500 cc volume, this filtration rate would yield a maximal increase in arm volume for

ten seconds of about 0.13 cubic centimeter. Lewis<sup>1</sup> found that venous pressure equaled a collecting cuff pressure of 49 mm of mercury in thirty nine seconds.

It seemed highly probable from the foregoing investigations that incoming blood could be the only significant factor responsible for the increase in arm volume which occurs during the first ten seconds after a collecting cuff is inflated about the human arm. However, we have been unable to find any direct evidence that such is the case. Hence the following experiment was performed.

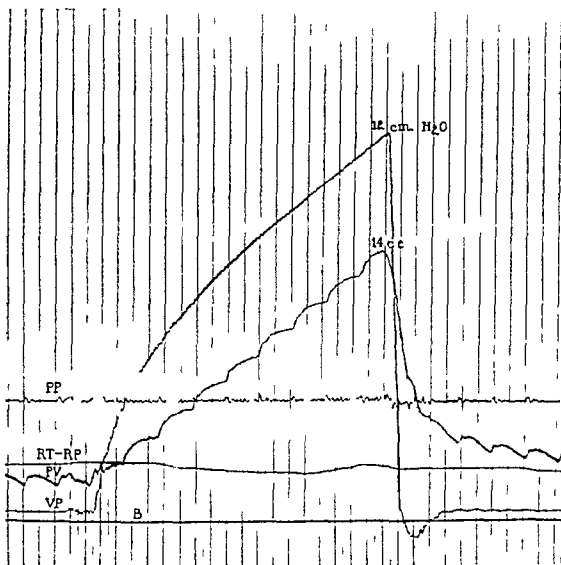


Fig. 10—Known flows of blood perfused through the isolated hind limbs of a dog by means of a heart lung preparation. PP, Pressure in the plethysmograph recorded by a Marey tambour. RT-RP, room temperature and room atmospheric pressure. VP, plethysmograph volume recorded by compensating spirometers. B, venous pressure in the hind limbs of the dog. B, base line. Heavy vertical lines, time in seconds; light vertical lines, time in one tenth second.

Using a small dog under pentobarbital sodium anesthesia a heart lung preparation was made of the upper half of the animal. Then the animal was transected in the midlumbar region. The abdominal aorta and vena cava of the lower half of the animal were cannulated and the entire lower half of the animal was sealed in the plethysmograph with a diaphragm occluding the outlet of the plethysmograph. An inlet for arterial blood and an outlet for venous blood from the lower portion of the animal were passed through the diaphragm and joined to the aforementioned cannulae in the abdominal aorta and vena cava. The lower half of the animal enclosed in the plethysmograph was then perfused

with blood by the heart lung preparation. Occlusion of the venous return was accomplished by clamping the venous outflow tube from the lower half of the animal. Thus flow curves were obtained. A simultaneous record of venous pressure distal to the point of clamping the venous return was made (Fig 10). Actual flows in cubic centimeters per minute were measured by collecting the blood as it emerged from the venous return tube. Estimation of the blood flow from the flow curve checked closely with the actual flow (Table II). We also noticed that venous pressure began to rise at the instant the venous return tube was clamped (Fig 10). Furthermore arterial inflow was unimpeded by a back venous pressure of about 17 cm. of water.

TABLE II CORRELATION OF ACTUAL BLOOD FLOW THROUGH HIND LIMBS OF DOG AND FLOWS CALCULATED BY THE PLETHYSMOKYMOGRAPHIC METHOD

ACTUAL BLOOD FLOWS CC MIN 100 CC				BLOOD FLOWS CALCULATED FROM FLOW CURVES		
FLOWS COLLECTED BEFORE FLOW CURVES WERE OBTAINED		FLOWS COLLECTED AFTER FLOW CURVES WERE OBTAINED		TEACING	MINIMUM	MAXIMUM
1	62	1	52	A	53.0	62.0
2	60	2	51			
3	58	3	55	B	59.0	62.5
1	16	1	17	A	14.5	14.5
2	48			B	13.0	13.0
1	18.5	1	45	A	46.0	50.0
2	18.5	2	11	B	18.5	18.5
	12		12		15.0	45.0
1	98	1	79		81.0	81.0
2	90					
3	80					
1	82	1	76	A	78.0	78.0
2	82			B	75.0	75.0
1	108	1	104	A	102.5	102.5
2	108			B	102.5	102.5

#### SUMMARY

A new recording device (compensating spirometer) was developed, analyzed, and compared with other types of recorders commonly used in plethysmokymography.

A new method for calibration of plethysmokymographs is described. Known flows actually collected in cubic centimeters per minute were compared with flows calculated from flow curves. When the compensating spirometer was used for recording blood flow, the discrepancy between actual and calculated values was small enough to permit the conclusion that this new device eliminates most of the errors inherent in other types of recorders and can be used for measuring blood flow in human extremities.

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## DEVICES FOR RAPID RECORDING OF MULTIPLE AND SPECIAL ELECTROCARDIOGRAPHIC LEADS

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PERHAPS the greatest advance in modern electrocardiography is the employment of special exploring leads (direct and semidirect) that are physically closer to a particular part of the heart and register more intimately its electrical activity. The theoretic importance of such leads was long recognized by workers in this field but for the sake of simplicity and convenience, so that electrocardiography could come into widespread use, the limb leads were emphasized as the exclusive standard. Now with the anterior surface of the heart being studied by multiple chest leads, the posterior surface and base by multiple esophageal leads, and the individual, separate chambers of the heart (such as the right auricle) by leads specially suited to the study, promise for real advance in electrocardiography is high. It is important, however, to determine what the particular usefulness of a special lead is and also which leads are so informative that they should by right be used routinely in electrocardiography, the added information will more than compensate for the extra effort in recording the tracings. Definite headway has been made in this regard, but much menial work such as obtaining normal standards for a given lead, comparing it with similar ones, and outlining its limits of usefulness still remains to be done. It was felt that this task could be expedited and made less unpleasant if special apparatus were designed to accomplish this type of multiple lead recording with speed. The apparatus to be described\* is flexible enough to record speedily almost every lead that has been proposed to date, and should be useful not only in this preliminary period when the standard of special multiple leads is being established, but also later in the routine recording of the multiple lead electrocardiogram which promises to be the tracing of the future.

*Switch Box*—The switching device which permits rapid recording of multiple leads without the moving of wires on the patient or the machine is shown in Fig 1. It consists of a main selector switch and two auxiliary switches which are interposed between the patient and the electrocardiograph. All manipulations for the selection of leads are accomplished at this switch, the electrocardiograph always recording the selected lead on its fixed Lead II setting. Five shielded wires run from the patient to the switch, one from each extremity (the right leg being used as a ground when the other three extremities are connected to the Wilson central terminal) and a fifth one from either the precordium or any other area to be explored. From the switch run the RA, LA, and LL wires of the electrocardiograph at connector marked ECG. Although only Lead II (RA and LL) is necessary for the recording, the switch makes all its grounding connections for any selected lead through its LA terminal. This insures a base line free of A C and other extraneous interferences, so that the switch box may be used with the newer types of direct writing electrocardiographs that are so sensitive to A C interference.

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\*Made by Medical Designs, Brooklyn, N Y.

The main selector switch permits recording not only of the conventional leads but also of the chest leads most commonly employed: precordium right arm (CR), precordium left arm (CL), precordium left leg (CF), and precordium Wilson central terminal (V, in which all three extremities are connected through equal 5,000 ohm noninductive resistances to furnish a point of zero potential).<sup>1</sup> There are also settings on the switch for obtaining the potential of the right arm, left arm or left leg, that is the unipolar limb leads with the Wilson terminal as the indifferent electrode ( $V_R$ ,  $V_L$ , and  $V_F$ ). The complexes obtained with the  $V_R$ ,  $V_L$ , and  $V_F$  leads tend to be small. Tracings of higher amplitude are secured by shunting out of the

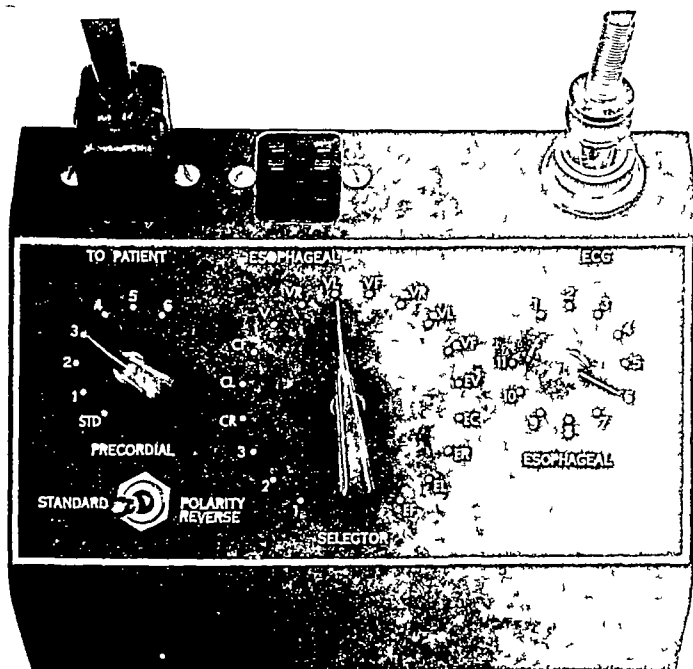


Fig 1—Switch box

circuit the 5,000 ohms resistance from the extremity that is being explored while keeping the resistors of the other two extremities in the Wilson hookup. The augmented Wilson unipolar leads can be recorded by turning the switch to the  $aV_R$ ,  $aV_L$ , or  $aV_F$  settings.

As seen in Fig 1, there are two adjacent switches used in conjunction with the main selector switch, the left hand one is for multiple leads taken from the front of the heart (precordial), and the right hand from the back of the heart (esophageal). For taking single precordial leads the exploring electrode (fifth patient wire) is used and the precordial switch is set at precordial (STD). If the multiple precordial lead belt, to be described later is used, the tracings from the six precordial electrodes are taken in succession by use of switch setting

1 to 6 inclusive. The main switch permits a choice of four indifferent points for precordial registration, that is, the right arm (CR), the left arm (CL), the left leg (CF), and the Wilson terminal (V). For esophageal tracings the eleven terminal esophageal lead, to be described later, is plugged into the selector switch and tracings from each of the eleven esophageal levels are taken in succession on the appropriate setting of the esophageal switch. Here there is a choice of five indifferent points for coupling with the esophageal lead: right arm ( $E_R$ ), left arm ( $E_L$ ), left leg ( $E_I$ ), precordium ( $E_C$ ) or Wilson terminal ( $E_V$ ). For the past thirteen years we have been accustomed to recording the esophageal lead with the exploring electrode connected to the RA terminal and the indifferent electrode to the LL terminal, so that the resultant tracing (taken the same way as Lead II) should resemble the

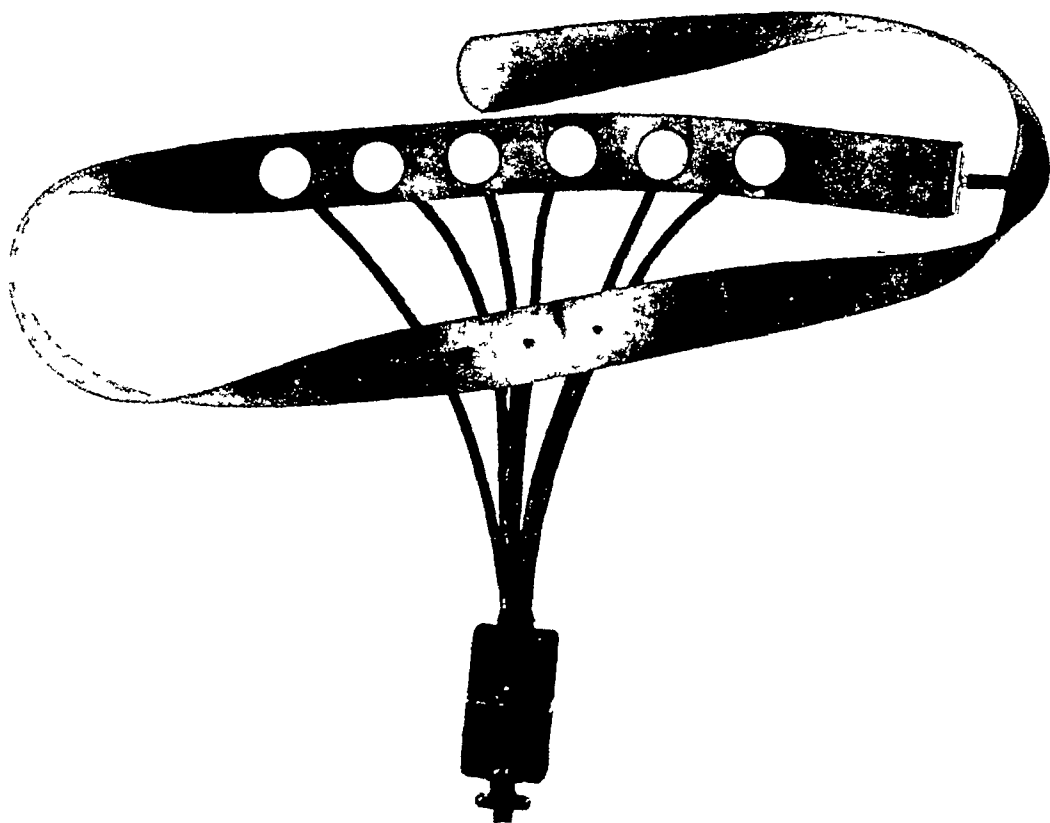


Fig. 2—Multiple precordial lead belt

precordial and conventional leads. This direction was followed by Brown<sup>3</sup> but is opposite to that used by Nyboer,<sup>4</sup> Graybiel and White,<sup>5</sup> and others. It is a simple matter to reverse the direction of the tracing by use of a simple throw switch marked Polarity Reverse on the left side of the switch box. This switch reverses the polarity not only of the esophageal lead, but also of any of the other leads when desired.

**Multiple Precordial Belt**—A modification of the elastic rubber belt described by Geiger and Goerner<sup>6</sup> has been found very effective in the rapid recording of multiple precordial leads (Fig. 2). The belt is made of high quality flat rubber belting about 1 inch wide and 42 inches long. Six flat German silver disk electrodes about 3 cm. in diameter are spaced 4 cm. apart toward one end of the belt. Soldered wires run from the disks through a six prong Jones connector and join the other five leads to form the eleven lead patient's cable (Fig. 3).

This cable plugs into the switch box at the connector marked To Patient. A hook on the free end of the strap fits into the proper hole punched in the belt making for easy adjustment of the elastic belt to the size of the chest. The only precordial placements that need be determined are the positions of electrodes 1 (right edge of sternum in the fourth interspace) and 6 (midaxillary line in the fifth interspace). The average technician locates these two points

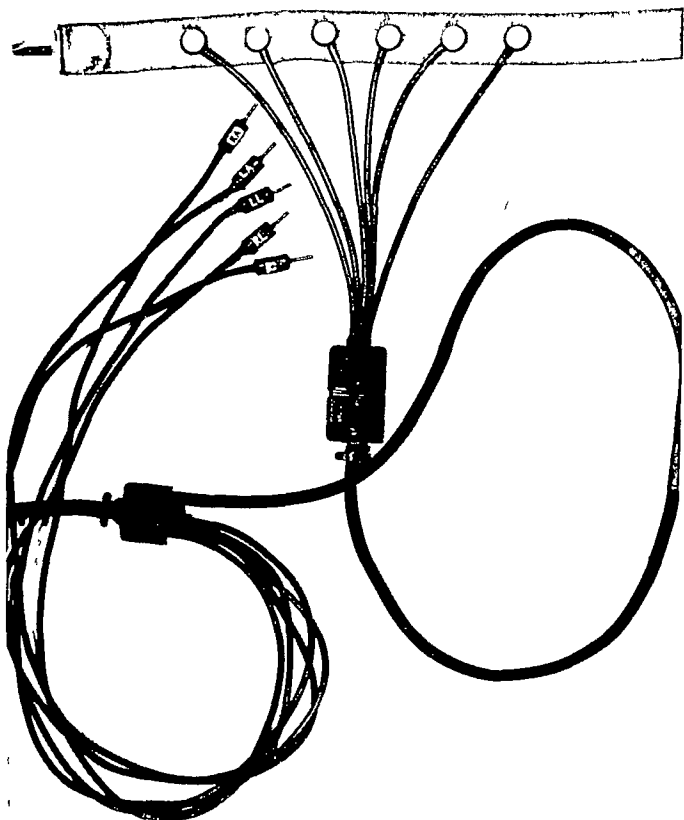


Fig. 3—Eleven lead patient's cable

easily enough. The other four electrodes lie automatically at equal distances from 1 to 6. The saving in time with this technique is appreciable. For still greater speed the following practical points may be noted. The rubber strap is laid across the arms of the electrocardiographic chair before the patient seats himself, avoiding the clumsy procedure of passing the belt around the patient's back. A thin layer of electrode paste is applied to



the six electrodes. The sixth electrode is first placed in the proper position. With the free end of the belt held in the left hand, the belt is put under tension with the right hand until electrode 1 has reached the proper position (right sternal edge). It is only then that the electrodes 1 to 5 are permitted to touch the chest. One can thus see the fifth disk falling into its proper position, then the fourth, the third, and so forth. If the amplifier type of electrocardiograph is used, no further rubbing of the skin is necessary. With the string type, the electrodes may have to be rubbed up and down to reduce skin resistance, particularly if the chest is hairy. The size of the female breast introduces no difficulty with this technique, for the breasts are always lifted and the belt is set in position beneath the mammary folds.

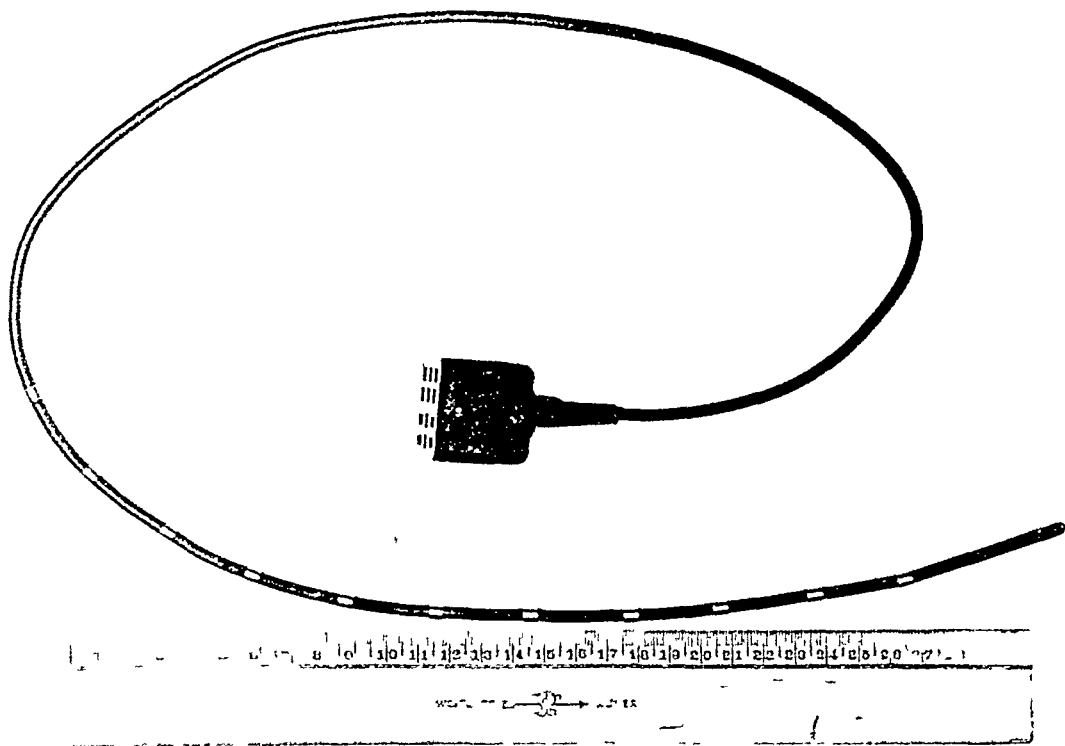


Fig 4—Eleven-electrode esophageal lead

*Esophageal Lead*—The esophageal lead designed for this study (Fig 4) is similar to Nyboer's.<sup>4</sup> The essential difference is that by an improvement in production technique an eleven-electrode lead can be built into a No. 12 Fr Levin tube, whereas to date this could be accomplished only in the larger 14 Fr or 16 Fr size. The 12 Fr tube can be readily passed, even in children. A special Levin tube which has no openings in the end is used so that no secretions can penetrate to the inside. Eleven narrow German silver rings spaced 3 cm apart are attached to the tube. A separate insulated wire leads from each ring through the tube to its appropriate post in a twelve-prong Jones connector plug. The twelfth prong of the plug connects to the electrical shielding of the cable that runs to the switch box (not shown in Fig 4). The technique in use with the esophageal lead is as follows. The patient is first reassured about the safety of the procedure. The rubber tip of the esophageal lead is lubricated for about an inch or two with a little mineral oil. Through the more patent

nostril the tube is rapidly passed beyond the posterior pharynx, which is likely to be the most sensitive part, down into the esophagus. The patient is asked to sip some water through a straw or drinking tube while the lead is being passed. This preoccupation with drinking permits the lead to be passed within about ten seconds to the proper level (the lowest electrode being placed 55 to 60 cm from the nares, as indicated by a mark on the tube). It is rarely necessary to use a local anesthetic for nose or pharynx. The patient is instructed to take a deep breath if uncomfortable or nauseated. In general, within a minute the patient will have become calm after the initial excitement of passing the tube. It will take, however, another five minutes or so for the esophagus to make its adjustment to the presence of the Levin tube in its lumen. During this time the position of the esophageal electrodes may be checked fluoroscopically if desired and the other connections can be made from the switch box to the patient's extremities and precordium. By the time the conventional leads and precordial leads have been recorded, the esophageal lead can be taken from each of the eleven esophageal levels in succession, using the esophageal switch. This five or ten minute waiting period after passing the esophageal lead, as well as the electrical shielding of the esophageal cable cuts down the drifting of the base line that is such a disturbing feature of esophageal electrocardiograms. It is to be noted that with the eleven electrodes spaced 3 cm apart the entire base and the posterior aspect of the heart are explored as far as the esophagus permits without the necessity of moving the esophageal lead up or down from its original placement. In contrast, most esophageal leads used until now have employed a single or only a few electrodes and have had to be manipulated up and down the esophagus to cover the electrical field desired. The consequent retching and esophageal contractions cause much drifting of the base line and numerous artifacts in the tracings. The amplifier type of machine is better suited in general for the recording of esophageal electrocardiograms because of the high resistances found in the esophagus. On the other hand we have taken very satisfactory tracings using the eleven electrode esophageal lead and a standard string galvanometer.

#### COMMENT

The devices outlined are being utilized in an extensive study of the usefulness of the many special and multiple leads suggested for the more intimate exploration of the heart and its separate chambers. Our findings will be reported at a later date when our data are more complete. It is considered worth while, however, to describe at this time the different devices designed to speed up the investigation, for similar studies must be made by numerous investigators and the findings correlated before a proper evaluation can be made of the usefulness of any proposed lead. The end in view of such studies is the establishment of the value of certain leads to the point where their inclusion in routine electrocardiography would be justified. There are other leads which, although not of such universal significance as to become routine may still be found most suitable for a special study of a particular part of the heart. Thus the exact usefulness and also the limitations of a derivation such as the esophageal lead, coming in contact with the left auricle and the base of the left ventricle, could be established. This type of study is tedious and time consuming, any device that simplifies the procedure appears to us worthy of description.

The main selector switch with its adjuvant multiple precordial and esophageal switches permits the rapid recording of practically all leads which have been proposed to date. The multiple esophageal and precordial leads can be connected further with a number of points as the indifferent electrode (right arm, left arm, left leg, Wilson central terminal, etc.), and the value of these different derivations can be compared. Although from the theoretic point of

view it may be best to couple all exploring leads with the Wilson terminal, empirically we, as well as others, have found instances in which other derivations were more informative. Whichever derivations will stand the test of time, it is likely that they can be easily registered with the multiple switch box described. Great pains have been taken to shield all the leads used in this study so that the devices could be employed with any electrocardiograph, even the direct-writing electrocardiographs that are so sensitive to A C interference. The machines that incorporate the instomatic principle of bringing the beam to the mid-line before switching to the next lead are particularly suitable to the rapid recording of multiple leads. Here one need only press the instomatic button and turn the multiple lead switch from one position to the other, and the machine is ready within a few seconds to record the new selection.

The technique described for taking multiple precordial leads is similar to the one used by Geiger and Goerner<sup>7</sup> and is advocated because of its simplicity and speed. Our experience with this belt technique almost exactly duplicates theirs. The difference between records obtained with this technique and the individual placements advocated by the American Heart Association<sup>8</sup> appear minimal and of little, if any, clinical significance. Results can be duplicated better with the belt than with the six individual placements, whether the physician or the technician determines them. Furthermore, in the belt technique, the fixed relation of the electrodes to the thorax rather than to the heart appears more sound, theoretically, in the study of many aspects of heart disease (axis deviation, bundle branch block, cardiac hypertrophy) than does the standard method.

The esophageal lead which we introduced in 1934<sup>2</sup> has not yet been widely employed, mainly because of technical difficulties. These have been overcome largely by the new design of the tube which can be passed easily enough to be used when indicated in any case except perhaps in terminal decompensation or during the first few weeks of a coronary occlusion. The switch which permits rapid recording from each of the eleven esophageal levels in succession saves so much time that the taking of an esophageal lead no longer can be looked upon as a formidable procedure. Once the physician convinces himself by actual trial that this is true, he can readily transmit this confidence to the patient, and the field of usefulness of the esophageal lead widens immeasurably.

#### SUMMARY AND CONCLUSIONS

Three devices designed for the rapid registration of multiple leads are described. The multiple switch box with a limb lead selector, multiple precordial lead selector, and multiple esophageal lead selector is the main device. Aside from the different chest leads (CR, CL, CF, and V), Wilson's unipolar limb leads and augmented unipolar limb leads can be obtained readily. A single precordial lead or six multiple precordial leads can be coupled with any of four indifferent points, and the eleven-electrode esophageal lead with any of five indifferent points.

An elastic multiple precordial lead belt is described which facilitates the rapid recording from six precordial areas by plugging into the switch box.

A thin eleven electrode esophageal lead which plugs into the selector switch is described. The procedure of recording an esophageal lead has been simplified so that it may be applied to any case except serious acute cardiac incidents.

It is hoped that these devices may prove of use to other investigators who are studying the comparative usefulness of special and multiple leads in electrocardiography.

We are deeply indebted to the late Dr. Frank Liberson and Dr. I. W. Held whose constant inspiration made this study possible.

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# DETERMINATION OF VOLATILE REDUCING SUBSTANCES (ALCOHOL OR ETHER) IN BLOOD AND GASES USING BARIUM DIPHENYLAMINE SULFONATE AS AN INDICATOR FOR CHROMIC ACID TITRATION

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IN THE determination of volatile reducing substances (primarily alcohol and ether) in blood or in air the usual procedure consists of absorbing these volatile organic substances in sulfuric acid, adding a known amount of potassium dichromate, and oxidizing the alcohol or ether to acetic acid by the chromic acid. The excess dichromate is then determined either colorimetrically or by titration.

With the colorimetric method as used by Gibson and Blotner (1938) and Newman and Abramson (1942), the organic substances are oxidized to acetic acid by chromic acid and the dichromate residue, consisting of chromic acid and chromic sulfate, is determined by absorption of light in the region of wave length 440 to 480 millmicrons. This method, while rapid, has the three following disadvantages: (1) The relation between the logarithm of the galvanometer reading and ether (or alcohol) concentration is not a straight line but is slightly convex. (2) Since light is absorbed by both dichromate and chromic sulfate it is necessary to calibrate the apparatus with ether (or alcohol) solutions of known concentration. Due to the highly volatile nature of ether and alcohol and the affinity of alcohol for water, the preparation, storage, and dispensing of standard solutions for precise work is a task requiring unusual precautions in technique. (3) For small quantities of blood the dichromate chromic sulfate mixture has low spectrophotometric sensitivity.

The titration method has been used widely. Excess dichromate after oxidation is titrated with a suitable reducing solution and a redox indicator. Iodometric titration of excess nonreduced dichromate was used by Shaffer and Ronzoni (1923), Newman (1936), McNally and Coleman (1944), Widmark (1922) and others, while Harger (1935), Cavett (1938), Levine and Bodansky (1939), and Fisk and Nelson (1941) preferred ferrous sulfate solution containing methyl orange. The methyl orange-ferrous sulfate solution was used in preference to the older iodometric titration because of the unsatisfactory titration end point of the older method.

In the present investigation the colorimetric method using the Evelyn colorimeter was used and was rejected for the reasons already given. Titration

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using the ferrous sulfate methyl orange mixture, was tried but rejected because the titration end point was not sharp. The redox indicator barium diphenylamine sulfonate was next investigated, using the titration procedure described by Kolthoff and Sandell (1938). This indicator, in the presence of phosphoric acid, gave a sharp end point with the color changing from violet blue to colorless. For titrating a sample of dichromate equivalent to one milligram of ether with 20 mls of a solution of ferrous sulfate a fraction of a drop of the reducing solution was sufficient to cause a sudden and striking change of color. This indicator was adopted for the determination of alcohol and ether in blood or in air with the procedure which will be described.

#### METHODS

**Reagents**—Ferrous sulfate, stock solution. Dissolve 50 Gm of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  in 150 ml of water. Add 30 ml of concentrated sulfuric acid and dilute to 250 milliliters. In a stoppered flask the solution will undergo only slight oxidation.

Barium diphenylamine sulfonate, stock solution. Dissolve 0.1 Gm barium diphenylamine sulfonate in 100 ml of water.

Ferrous sulfate indicator solution for titration. To 15 ml of the ferrous sulfate stock solution and 20 ml of the indicator stock solution add water to 2000 milliliters.

Potassium dichromate standard solution. Weigh out exactly 5.295 Gm  $\text{K}_2\text{Cr}_2\text{O}_7$  (Mallinckrodt's analytical reagent), dissolve in water and dilute to one liter. One milliliter of this solution is equivalent to 1 mg ether.

Sulfuric acid, C P Concentrated

Phosphoric acid, C P 85 per cent

**Procedure for Determining Ether in Blood**—Widmark flasks (Cavett 1938) of 50 ml capacity with T 24 standard tapered Pyrex stoppers were made (Fig 1, A). It is desirable in preparing these flasks to make the glass rod by means of which the cup is suspended from the stopper as short as possible. This prevents the cup (2.5 ml capacity) from dipping into the chromic acid at the bottom of the flask.

To determine ether in blood the blood is drawn into a syringe with a drop of saturated potassium oxalate filling the needle and needle tip of the syringe. The blood is drawn with care to exclude bubbles in the syringe and is discharged into a two way stopcock pipette which contains approximately 0.25 ml of water in one arm (Fig 1, A). This pipette is made to contain approximately 1 ml in the lower arm. The volume of the lower arm is carefully determined with mercury or standard solution. Blood is discharged from the syringe as shown in Fig 1, A, and it ascends in the lower arm through the stopcock to the upper arm which does not contain the water. Approximately 0.5 ml of blood will ascend above the stopcock. The blood exposed to air as it ascends in the pipette will have lost ether to the air above its surface. This blood will be rejected. The stopcock is now closed, the syringe removed from the pipette and the lower

tip of the pipette held above the cup of the Widmark flask. The stopcock is turned to the arm containing the water, the blood is drained into the Widmark cup and is washed from the pipette with the 1 ml of water. The Widmark flask is stoppered immediately. A syringe pipette also has been used and found to be satisfactory.

Either 10 or 15 ml of standard dichromate solution from a dispensing burette are placed in the bottom of the Widmark flask and 30 ml of concentrated sulfuric acid are added. Similar quantities of dichromate and sulfuric acid are measured out for a blank and placed in a glass-stoppered 50 ml Erlenmeyer flask. The blood and wash water are placed in the Widmark cup as

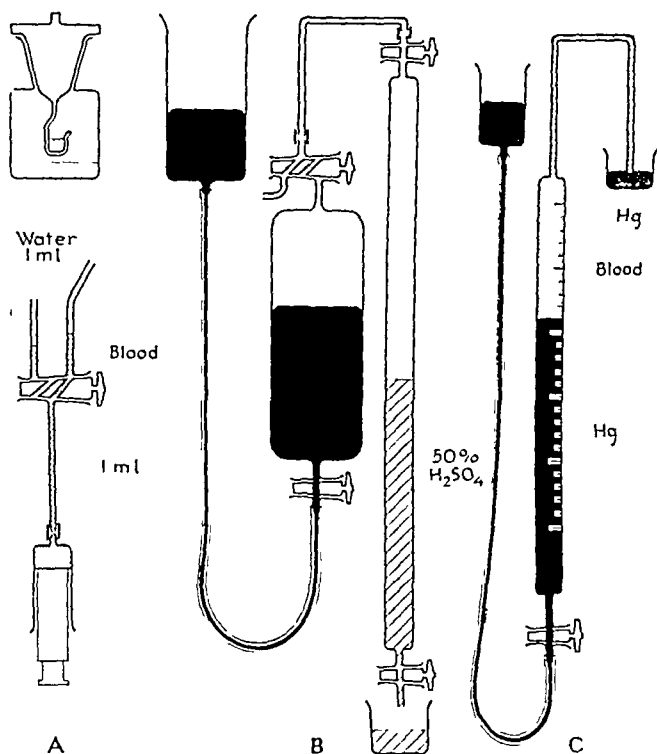


FIG. 1—A, Blood pipette and syringe; Widmark flask; B, gas sampling bulb and ether absorber; C, burette for storage and dispensing of solutions with volatile solutes.

described and both the Widmark flask and the Erlenmeyer flask transferred to an oven at 80° C for four hours. The solutions are removed then and it will be observed that the blood has hardened to a cake by evaporation. After cooling, 3 ml of concentrated phosphoric acid are added and the solutions are titrated in the same flasks with ferrous sulfate containing diphenylamine sulfonate. The number of milligrams of ether in 100 ml of blood is given by the formula

$$\text{Ether (mg) in 100 ml of blood} = \frac{T_R - T_N}{T_R - 3e} \cdot \frac{100}{V} \cdot N$$

where  $T_B$  is the titration value of the blank (milliliters ferrous sulfate solution)  $T_x$ , the titration value of the unknown,  $V$ , the volume of the sample to be analyzed,  $N$ , the number of milliliters of the standard dichromate solution, and  $e$ , the reducing equivalent in milliliters of ferrous sulfate solution of 1 ml of concentrated sulfuric acid which is determined as explained later

*Procedure for Determining Ether in Air* —The air ether mixture is collected in a gas sampling bulb of approximately 60 ml whose exact volume is determined by calibration. The sampling bulb has a one way stopcock at the bottom and a two way stopcock at the top and is connected to the bulb as shown in Fig. 1 B. The upper stopcock of the gas sampling bulb is joined with a short rubber connection to a glass capillary tube which is then connected to a long 100 ml tube (an old 100 ml burette can be used for the purpose). The 100 ml tube has a stopcock at the top and bottom, and at the start is filled by suction to the upper stopcock with 50 per cent sulfuric acid. The gas from the sampling bulb is transferred to the long absorber tube and displaces the sulfuric acid. After all the gas has been transferred approximately 10 ml of ether free air are washed from the stopcock at the top of the sampling bulb through the connecting tube to the absorber. The stopcocks at the bottom and top of the absorber are closed the connecting tube is disconnected from the sampling bulb and the absorber is shaken for about one minute and allowed to stand ten minutes. The contents of the absorber are then transferred to a 50 ml volumetric flask, the absorber is washed with approximately 5 ml of 50 per cent sulfuric acid which is transferred to the volumetric flask and the flask is made up to 50 ml with 50 per cent sulfuric acid. Three milliliters of the contents of the volumetric flask are placed in a glass stoppered Erlenmeyer flask containing 1.50 ml of the standard dichromate solution, the flask is allowed to stand at room temperature for thirty minutes. This solution and a flask containing 3 ml of 50 per cent sulfuric acid and 1.00 ml of standard dichromate are titrated at the same time. The amount of ether in 100 ml of the air ether mixture is computed from the following formula

$$\text{Ether (mg) in 100 ml of air ether mixture} = \frac{T_B - T_x}{T_B - 1.5e} \cdot \frac{100}{V} \cdot \frac{50}{N}$$

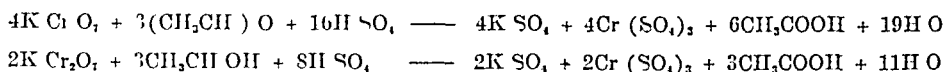
where  $T_B$  is the titration value of the blank in the flask  $T_x$ , the titration value of the unknown,  $e$  the reducing equivalent of the sulfuric acid used  $V$  the volume of the gas sampling tube in milliliters  $N$  the number of milliliters of dichromate, the volume of the volumetric flask 50 ml and the pipette volume 3 milliliters

*Test for Reducing Power of Sulfuric Acid* —Sulfuric acid contains a variable amount of reducing material, possibly  $SO_2$  which reduces ferrous sulfate. In many samples of sulfuric acid there are no reducing substances. For accurate analyses an estimate must be made of the reducing power,  $e$ , of the sulfuric acid used in the procedure. This is done by placing in a series of six Erlenmeyer flasks 1 ml of the standard dichromate and 0, 1, 2, 3, 4, and 5 ml of



concentrated sulfuric acid To each flask, 3 ml of concentrated phosphoric acid are added and the solutions titrated The titration values are plotted against milliliters of sulfuric acid, and the reducing power,  $e$ , per milliliter of sulfuric acid determined This titration must be made for each new bottle of sulfuric acid used The value of  $e$  has not exceeded 0.5 ml of the ferrous sulfate solution used for titration All samples of phosphoric acid tested were found to be free of reducing substances

*Test for Oxidation of Acetic Acid*—In the NiClour oxidation of ether and alcohol, as given by the following equations, two assumptions are made, namely (1) that all of the alcohol (or ether) is oxidized to acetic acid, and (2) that none of the acetic acid is oxidized



If these assumptions are valid, the oxidation proceeds as shown by the equations and an equivalence can be computed whereby 1 milligram of ether is equivalent to 5.295 mg of potassium dichromate and 1 mg of alcohol is equivalent to 4.259 mg of potassium dichromate It is necessary to determine, as pointed out by Shaffer and Ronzoni but ignored by many later workers, whether or not oxidation is complete and whether or not acetic acid is oxidized A number of experiments were performed to determine whether acetic acid was oxidized under the conditions of the experiment A solution of acetic acid containing 1.68 mg of acetic acid per milliliter of solution was prepared and tested for oxidation Four solutions, each containing 1 ml of standard dichromate solution and 3 ml of concentrated sulfuric acid, were prepared (in quadruplicate) and treated in the following way (A) Blank—titrated with ferrous sulfate—diphenylamine sulfonate solution at room temperature, (B) 1 ml of acetic acid solution added and titrated at room temperature, (C) Blank (chromic acid alone), heated for four hours at 80° C, (D) 1 ml of acetic acid solution added and heated in an oven for four hours at 80° C The results are contained in the following table

SOLUTION	CONDITION	TITRATION VALUE	EQUIVALENT ETHER (MG)
A Blank	room temperature	23.80	1.00
B Blank with 1 ml of acetic acid	room temperature	23.83	1.00
C Blank	80° C for 4 hr	23.09	1.00
D Blank with 1 ml of acetic acid	80° C for 4 hr	22.99	1.009

It is to be noted that the oxidation of acetic acid equivalent to 1 mg of ether is less than 1 per cent in terms of the error involved in ether analysis, and hence is so small that it can be neglected However, on comparing A and C it is to be noted that there is a loss of approximately 3 per cent in the oxidizing power of chromic acid when kept for four hours at 80° C This result requires that the blank chromic acid be treated in the same manner as the chromic acid which absorbs ether, namely being kept with the ether absorbing solution at 80° C for four hours

**Preparation of Standard Solutions of Ether and Alcohol**—The method used for the preparation of standard solutions of ether and alcohol is as follows:—A certain amount of pure ether or alcohol is weighed and dissolved in a known volume of water. For example, 10 mg of ether per milliliter of water. The standard solutions are then used for the determination of ether in blood. The standard solutions are difficult to prepare due to the high volatility of ether and alcohol. The standard solutions are difficult to store due to the high volatility of ether and alcohol.

The best method of storing and dispensing the standard solutions was found to be as follows:—A certain amount of pure ether or alcohol is weighed and dissolved in a known volume of water. For example, 10 mg of ether per milliliter of water. The standard solutions are then used for the determination of ether in blood. The standard solutions are difficult to prepare due to the high volatility of ether and alcohol. The standard solutions are difficult to store due to the high volatility of ether and alcohol.

**Test for Recovery From Prepared Solutions of Ether in Blood**—A recovery test from prepared solutions of ether in blood was made. The results are as follows:—A certain amount of pure ether or alcohol is weighed and dissolved in a known volume of water. For example, 10 mg of ether per milliliter of water. The standard solutions are then used for the determination of ether in blood. The standard solutions are difficult to prepare due to the high volatility of ether and alcohol. The standard solutions are difficult to store due to the high volatility of ether and alcohol.

Organic volatile matter in 100 ml blood was 2.5 milligrams

Corrected value for ether in blood was 162.5 mg per 100 milliliter. An error of 2 per cent was involved which was probably due to the errors involved in preparation of the standard solutions.

**Distillation Time**—A test was made to determine the time required for distillation in the oven at 40°C. The time will vary with the surface area and volume of the two solutions in the Wigglesworth flask. The time required for distillation in the cup and the receiving solution (chromic acid) at the bottom of the flask. Following are the data on recovery of ether from a standard solution of ether in blood.

2 hours in oven	2 per cent
3 hours in oven	75.5 per cent
4 hours in oven	99.2 per cent

With the Widmark flasks used, four hours in the oven were required for complete distillation although distillation was 90 per cent complete after two hours. Distillation is not complete until the blood sample is hard and caked.

#### DISCUSSION

The Widmark method of determining ether (or alcohol) in blood has the advantage of eliminating a distillation process, which permits a considerable saving of time. It does require, however, a wait of four hours for ether to diffuse from the solution in the cup to the receiving solution at the bottom of the flask. Where speed in analysis is not essential, the convenience of this method is an advantage. The four-hour period in the oven will cause a loss of the oxidizing power of the dichromate which will amount to as much as 5 per cent. This can be corrected for by placing the blank chromic acid in the oven with the ether sample. The method used by Levine and Bodansky (1940), in which the sample of blood containing alcohol was absorbed by a filter paper and suspended within a flask containing chromic acid, was found to be unsatisfactory due to the dropping of invisible particles of dust or dry material from the paper into the chromic acid (Anderson, 1942). Rapid transfer is effected by this method but irregular results are obtained. In using chromic acid for absorption of less than milligram quantities of ether, it is necessary to use scrupulously clean glassware and to avoid dust particles. All open flasks must be covered and only the cleanest of stirring rods, kept dust free, can be used. It was the customary practice in a series of titrations to discard the first blank titration which was usually low, probably due to dust. If rapid analysis is necessary and if the delay due to ether transfer in the Widmark flasks is to be avoided, a distillation similar to that described by McNally and Coleman (1944) could be used.

A precaution which is recommended is that chromic acid cleaning solution be avoided in cleaning the glassware. Trisodium phosphate cleaning solution has been found to be satisfactory for this purpose.

The reagents to be dispensed, including ferrous sulfate, sulfuric acid, phosphoric acid, and standard dichromate, are all delivered from burettes. A 2 ml burette is used for the standard dichromate, and 50 ml burettes are used for the concentrated acids. These acids are delivered to the burettes from an all-glass reservoir and connecting system by pressure. Phosphoric acid is used as a stopcock lubricant for the burettes containing the strong acids, but is not highly satisfactory since a slight sticking of the stopcocks will occur. Some of the silicone greases might be useful for this purpose. A minimum amount of grease must be used on the stopcocks of the burettes containing the potassium dichromate and the ferrous sulfate.

In using the redox indicator, barium diphenylamine sulfonate, the indicator can be added in a constant amount to the dichromate solution. The indicator itself undergoes oxidation and reduction, hence a correction is theoretically required. This correction cancels out and the step in the analysis which requires addition of the indicator can be eliminated if the indicator is added to the

ferrous sulfate reducing solution. The total reductant then includes both the indicator and the ferrous sulfate and the mixture is standardized against a known amount of potassium dichromate. This procedure has the disadvantage that when only a small amount of chromic acid remains unreduced in the solution and only a few milliliters of reducing solution are needed the amount of indicator present may be insufficient to produce a well defined color. This difficulty can be avoided by adding sufficient excess dichromate which is always a safe procedure in any event since if the amount of dichromate is insufficient for the ether present the analysis is spoiled. An estimated excess of 0.2 to 0.5 ml of standard dichromate which required 4 to 10 ml of the ferrous sulfate indicator solution was found to be satisfactory.

In evaluating the present method in comparison with other methods described in the literature (Levine and Bodansky, 1939) the present method is found to be unique in possessing a sharp well defined and highly chromogenic end point. The titration method relies for standardization and calibration on a single standard solution of potassium dichromate which is a reagent readily available in a highly purified form and therefore makes an excellent standard. It is not necessary as is required with the colorimeter method to calibrate with standard solutions of alcohol or ether. These are required in the colorimeter method because the color developed after reduction of part of the chromic acid by alcohol or ether is due to both dichromate and chromic sulfate. Furthermore with the colorimeter method it is not possible to use the completely oxidized dichromate and the completely reduced dichromate that is chromic sulfate as limiting values on a calibration graph since the calibration curve of logarithm of colorimeter reading plotted against ether (or alcohol) concentration is not a straight line and the deviation is greatest at the limits.

#### SUMMARY

A method for determining ether (or alcohol) in blood and gas mixtures is described. The method consists of a distillation in a Widmark flask with oxidation of the transferred alcohol or ether by chromic acid. Excess chromic acid is titrated with ferrous sulfate using barium diphenylamine sulfonate as an indicator.

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## RECTAL ABSORPTION OF PENICILLIN

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SINCE the early days of the penicillin era destruction by fecal enzymes has been regarded as one of the main obstacles to effective low intestinal absorption of penicillin.<sup>1-10</sup> Results of more recent investigations on this subject are contradictory. Absorption from ligated loops of the colon of rats was determined<sup>11</sup> to be nearly the same as that from the duodenum. Other authors<sup>6</sup> reported the penicillin uptake from isolated segments of the large intestine of rats to be inferior to duodenal absorption. In a man with a fistula of the colon penicillin activity in blood and urine was negligible (23 per cent urinary recovery) after introduction through the fistula of 0.3 million units in solution.<sup>6</sup> In apparent contrast to these observations Loewe and associates<sup>12</sup> obtained therapeutic serum concentrations with the rectal use of penicillin suppositories (up to 0.77 unit per milliliter one hour after insertion of one million units). The statement by Drayson<sup>13</sup> that 'rectal suppositories will maintain a therapeutic level for twenty-four hours' is probably based on those findings. Stimulated by this same report Baruch and co-workers<sup>14</sup> introduced penicillin in aqueous solution rectally only to find no demonstrable blood level. Nevertheless, a paper from the Mayo Clinic<sup>1</sup> which appeared while the present study was in progress listed results comparable to Loewe's, as produced with penicillin suppositories.

In view of these discrepancies and because of the lack of conclusive information on quantitative penicillin absorption from the rectum the present work was undertaken.

### EXPERIMENTS AND METHODS

All experiments were performed on adult male human subjects between the ages of 18 and 50 and on two children as indicated below, all of whom had no apparent organic intestinal disorder. Penicillin was administered rectally in cocoa butter suppositories, cocoa butter capsules, gelatine capsule and by microclyster and insufflation and the resulting antibiotic activity of blood and urine was determined. For reasons of comparison parenteral and oral administration were also studied.

The suppositories (Table I) were made by mixing dry penicillin with cocoa butter at 50 to 60° C. to which was added a small amount of peanut oil or kaolin in the first few trials and 2 per cent beeswax in the later ones. The streptomycin suppositories used in four

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trials (Table I, Cases 12 to 15) were prepared in the same manner, two such suppositories, each containing 0.25 Gm., were inserted two hours before a penicillin suppository. A cleansing saline enema was given in this and in the subsequent group of experiments one to three hours before the test to those subjects in whom satisfactory elimination had not occurred earlier the same morning. No such enemata were used in any of the following experiments.

A microclyster (Table II) was prepared by dissolving penicillin in physiologic saline (100,000 units per 5 ml.). Injection into the rectum was performed through a rubber catheter, the patient remaining on the side in order to prevent leakage.

Cocoa butter capsules were made by placing dry penicillin in the center of the cold mold which had been covered by a layer of hardened cocoa butter. One or two drops of the melted oil closed the opening, forming the base of the suppository capsule which was then kept under refrigeration until immediately before administration (Table III).

Insufflation of penicillin was carried out through an anoscope into the rectum of five subjects (Table VI). A small amount of water-soluble lubricant was applied to the outside of the instrument before its introduction.

For penicillin assay as shown in Tables I, II, and V, the Food and Drug Administration cup plate method<sup>16</sup> was employed in the urine and a modification of Rummelkamp's method<sup>1</sup> in the serum\*. In the latter determination, the susceptibility of the lyophilized hemolytic streptococcus strain used as the inoculum averaged 0.015 unit per milliliter.

Penicillin assay listed in all other tables (Tables III, IV, VI, and VII) was performed by means of (1) a modification of Fleming's micromethod<sup>18†</sup> and (2) a serial dilution method similar to Kolmer's<sup>19</sup> employing the *Staphylococcus aureus* strain 209 P as the test organism‡. A number of specimens were subjected to parallel tests by either method, as indicated in the tables by two figures representing one specimen.

Streptomycin in the urine was determined according to the method of the Food and Drug Administration<sup>20\*</sup>.

## RESULTS

Absorption of penicillin from a suppository was prompt and rapid (Table I), the highest blood level in every case was found in the first blood specimen taken, the fifteen-minute level, whenever obtained, exceeded the half-hour reading which in turn was invariably higher than the result of the one-hour sample. Most significant in this connection was the occurrence (Case 5) of sudden diarrhea ten minutes after the suppository was inserted, the blood titer five minutes thereafter, as well as the penicillin excretion in the six-hour urine specimen, compared well with the respective findings in other trials in which no such loss was observed. Penicillin activity of the serum was still well assayable after six hours whenever determined in Cases 1 to 11, and after nine hours in Cases 8 and 11 (two subjects receiving high dosage). In all instances in which fractional urine samples were examined, the quantity of penicillin excreted in the first three hours exceeded the amount found in the following nine hours and amounted in most cases to more than 50 per cent of the twenty-four hour recovery. The latter ranged between 3.6 and 8.3 per cent, a mean percentage recovery of 6.3.

\*These assays were carried out at the Venereal Disease Research Laboratories, U. S. Marine Hospital, Staten Island, N. Y.

†Carried out at the Department of Biochemistry, Northwestern University Medical School, Chicago, Ill. (Chief Dr. C. J. Farmer).

‡Unpublished method by Miss Helen MacLean, Department of Bacteriology, Michael Reese Hospital, Chicago, Ill.

TABLE I SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING RECTAL ADMINISTRATION OF AMORPHOUS SODIUM PENICILLIN BY COCOA BUTTER SUPPOSITORY

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML)					PERCENTAGE OF DOSE EXCRETED		
		HOURS					HOURS		
		1/4	1	1	3	6	3	1	24
1	0.5			0.125	0.03	0.033 (7 hr)	-	45	57
2a	0.5			0.107	0.025	0.018	-	74	75
3b	0.5	0.833	0.75	0.214	0.03		68	70	70
4c	0.5	0.214	0.187	0.075	0.025	0.025	36	42	42
5d	0.5	0.37					-	33 (6 hr)	-
6	0.5		0.545	0.075			78	-	-
7	0.5		0.214	0.15	0.015		38	-	-
8	1.0		0.5	0.187	0.10	0.093	-	52	83
9	1.0			0.3 (1 1/2 hr)	0.042	0.015	-	63	74
10	1.0	0.75	0.6	0.15	0.05	0.042	23	-	45
11d	1.0	1.2	0.75	0.3		0.107	25	49	54
12a	0.5 (2 hr after streptomycin)		0.166	0.018	0	0	25	-	30
13c	0.5 (2 hr after streptomycin)		0.125	0.08	0	0	17	2	31
14b	0.5 (2 hr after streptomycin)	0.5	0.187	0.136	0.015	0	47	61	61
15	0.5 (2 hr after streptomycin)	0.05	0.025	0	0	0	08	16	16

Letters a b c and d indicate same patients subjected to different experiments

0 Less than the minimal amount of penicillin inhibiting the test organism

Serum level at nine hours in Case 8 0.093 Case 9 0 Case 11d 0.03

The rectal application of streptomycin preceding the insertion of a penicillin suppository (Table I, Cases 12 to 15) caused an apparent lowering of penicillin values in both blood and urine. This fact is particularly obvious when the results in Cases 12 to 14 are compared with those obtained in the same patients without the use of streptomycin (Cases 2 to 4). After three hours penicillin activity of the blood was at or below the sensitivity limit of the test organism.

In all urine specimens collected in Cases 12 to 15 the streptomycin content was either minute (Case 14) or not demonstrable a result expected from investigations of oral ingestion.<sup>1</sup>

In the retention enema group (Table II) penicillin activity in the serum tested in each case in as many samples as in the suppository series was found to be either very low (maximum was 0.05 unit per milliliter at six hours in Case 4) or not assailable at all. Also the total urinary recovery was generally less in the saline group. The quantity of penicillin excreted during the first collection period was usually smaller than in the subsequent sample in contrast to the predominantly inverse ratio in the former series.

The findings in cases treated with cocoa butter capsules (Table III) were in part similar to those experienced in the suppository trials with early peaks of the blood levels and a penicillin excretion predominant within the first few



TABLE II PENICILLIN EXCRETION IN URINE FOLLOWING ADMINISTRATION OF AMORPHOUS SODIUM PENICILLIN BY RECTAL MICROCLASTER

CASE	DOSAGE (MILLION UNITS)	PERCENTAGE OF DOSE EXCRETED		
		HOURS		
		3	12	24
1	0.1	1.6	2.5	2.5
2	0.5	0.3	1.0	1.1
3	0.5	0.5	1.5	1.5
4e	0.5	0.3	5.0	8.7
5e	1.0	0.2	0.8	1.2

e Same patient subjected to different experiments

TABLE III SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING ITS RECTAL ADMINISTRATION BY COCOA BUTTER CAPSULE

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML.)			PERCENTAGE OF DOSE EXCRETED	
		HOURS			HOURS	
		1/4	1/2	3	3	6.21
1	0.2 amorphous sodium		2.0*		24.8	30.4
2	0.2 amorphous calcium	8.0*			23.8*	22.5
3	0.2 amorphous calcium	2.56		0.08	15.4	16.8
4†	0.2 crystalline sodium	4.0*	1.28	0.08	25.6	26.2
5†	0.5 crystalline sodium	12.8	6.4	0.4	28.8	35.2
			(1 hour)			
6§	0.1 amorphous sodium	2.56		0.04	14.5	-
7§	0.2 crystalline sodium G	5.12		0.16	17.2	18.1

\*Results determined at Northwestern University

†Defecation occurred ten minutes after insertion of capsule

‡Seven-year-old boy weighing 56 pounds Serum level at five hours 0.32

§Six-year-old boy weighing 45 pounds

hours. The excretion ratio of 26.2 per cent in Case 4, where defecation occurred ten minutes after insertion of the capsule, deserves special attention. The greater height of the serum levels and the average urinary recovery ratio of about 25 per cent make this method far superior to the simple suppositories.

With the use of rectal gelatin capsules\* (Tables IV and V), the urinary penicillin excretion was generally lower and within a much wider range, allowing no mean value to be computed. The serum concentrations likewise were lower than those shown in Table III. The half-hour readings in Cases 1 and 4 were higher than those of the earlier levels, and later urine specimens often contained more penicillin than the first three hour collections, suggesting a relative delay in absorption.

In the insufflation experiments, the actual amount of penicillin reaching and adhering to the mucosa was surely less than the quantity obtained from the commercial vials, since diffusion of some of the powder over the inner surface of the instrument and over the examiner's face and clothing was noted following every such procedure. Therefore, the true absorption ratio for the penicillin which remained in the rectum is undoubtedly better than appears from Table VI. Still, most results here are comparable with those found with cocoa butter

\*Gelatin capsules Rectal No. 1 Parke Davis &amp; Company Detroit Mich.

TABLE IV SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING ITS RECTAL ADMINISTRATION BY GELATIN CAPSULE

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML)					PERCENTAGE OF DOSE EXCRETED	
		HOURS					HOURS	
		1/4	1/2	1	-	3	3	15 24
1	0.2 amorphous calcium	0.08	1.28				5.3	14.0
2	0.2 amorphous calcium		0.25*			0	2.3*	10.3
3	0.2 amorphous calcium		0.4				9.2	13.5
4	0.5 crystalline sodium	2.56	5.12		0.9		18.0	22.7
		2.0*	4.0*		1.0			
5†	0.2 crystalline sodium		0	0.12		0	18.2	-
6†	0.5 crystalline sodium		3.2	0.4		0.08	-	-

\*Results determined at Northwestern University

0 Less than 0.06 which is the sensitivity limit of the test organism used

†Same child as in Case 5 Table III

TABLE V PENICILLIN EXCRETION IN URINE FOLLOWING RECTAL ADMINISTRATION OF 0.2 MILLION UNITS OF CRYSTALLINE SODIUM PENICILLIN BY GELATIN CAPSULE

CASE	PERCENTAGE OF DOSE EXCRETED		
	HOURS		
	3	5	24
1	3.7	4.8	7.2
2	1.2	4.3	5.1
3*	0.3	0.6	1.0
4	0.1	4.1	5.8
5	8.7	10.7	18.0

Defecation occurred at the sixth hour

TABLE VI SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING RECTAL INSUFFLATION OF 0.2 MILLION UNITS OF AMORPHOUS CALCIUM PENICILLIN

CASE	SERUM LEVELS (UNITS IPR ML )			PFRCENTAGE OF DOSE EXCRETED		REMARKS
	HOUPS			HOURS		
	1/4	1/2	3	3	6 20	
1		1.28		30.7*	34.5*	Fecal scybala visible in rectum
		1.0*				
2	>16		0.08	14.7	-	Insufflation done after liquid def
	8.0			22.1*	22.3	ecation much backfire
3	8.0			33.3	-	Scybala visible in rectum
				49.9*		
4	2.0			15.3*	17.4*	Marked diffu ion
5	0.64		0	5.4	5.7	Some fecal matter visible in rec tum marked diffusion

Results determined at Northwestern University

0 Less than 0.04 the sensitivity limit of the test organism

TABLE VII SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING ADMINISTRATION OF CRYSTALLINE SODIUM PENICILLIN BY INTRAMUSCULAR INJECTION OF AN AQUEOUS SOLUTION AND BY INGESTION IN A GELATIN CAPSULE ONE HALF HOUR BEFORE NOON MEAL

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML)			PERCENTAGE OF DOSE EXCRETED	
		HOURS			HOURS	
		1/4	1/2	3	3	24
1	0.1 (intramuscularly)	10.24		0.16	69.2	70.9
		8.0		0.25*		
2†	0.5 (orally)	2.56	1.6	0.08	39.4	43.8

Results determined at Northwestern University

†Same child as in Case 5 Table III

capsules in regard to both speed and degree of systemic uptake. The presence of much fecal matter (Cases 1 and 3) did not seem to interfere noticeably with absorption.

#### DISCUSSION

Rectal absorption from a penicillin solution is more erratic and less prompt and efficient than from a suppository or cocoa butter capsule containing the drug. The reason for this could be passage of enemas into the proximal portions of the colon<sup>22</sup> with more limited absorption capacity and exposure of penicillin in the aqueous medium to bacterial penicillinase action. On the other hand, a suppository or capsule remains in the lower rectum just above the sphincters where absorption is enhanced by the hemorrhoidal venous plexus with its portal and caval communications<sup>23</sup>, besides, penicillinase activity is unlikely in an oily medium<sup>24</sup>. This distinction in absorption between penicillin suppositories and enemas appears both to confirm and explain the contradictory experimental reports referred to.

The rapid initial absorption from a simple suppository associated with a relatively low blood level and urinary excretion, when first observed, indicated a retention in the rectum of most of the drug. The early descent of the blood level was probably not due to penicillinase interference, since it takes several hours to record in *in vitro* emulsions of stools with penicillin a marked loss of antibiotic activity.<sup>6</sup> Moreover, the streptomycin suppositories given in order to combat colon bacillus activity and to increase penicillin absorption were found to depress the latter (Table I). These facts pointed to the accumulation in the rectum of the oily material and to its intimate contact with penicillin as important interfering factors. The experiments with insufflation and with cocoa butter capsules, containing the vehicle in smaller quantity and unmixed with the antibiotic, seem to have borne out these conclusions, the efficiency of absorption here is about fourfold.

The mostly disappointing results with gelatin capsules may be accounted for by slow or incomplete disintegration of gelatin and/or by possible adsorption of penicillin to the dissolved gelatin or to the lubricant used in some instances.

The insufflation experiments, inaccurate as they may be, suggest that absorption from pure dry penicillin applied directly to the rectal mucosa is prompt and therapeutically significant. No noteworthy local irritation was observed in these or in any other of the experiments reported.

The type of penicillin used, as indicated in the tables, does not seem to have had a noticeable bearing on the results.

The accuracy of all the findings tabulated, save for the saline group, is impaired by the inevitable loss of penicillin incurred in the preparation of the various suppositories and capsules, estimated at 5 to 10 per cent. Dosages as listed in the tables are, of course, copied from the labels of the vials from which the drug was obtained. The true serum and excretion values, therefore, in each such experiment must be regarded as somewhat higher than tabulated. Needless to say, the error inherent in the methods of bio-assay employed is considerable ( $\pm 20$  per cent).

In any case, the average excretion ratio of penicillin applied rectally either by insufflation or in cocoa butter capsules appears to amount to no less than one fifth of the absorption coefficient of injected penicillin (Table VII), which ranges between 60 and 100 per cent<sup>7-9</sup>. Thus the rectal route is not inferior to the oral route which produces an average penicillin absorption of 20 per cent<sup>6,7,28</sup>. This is also evident from a comparison of the results of oral administration (Table VII, Case 2) with those of rectal administration (Table III, Case 5) in the same child. With the other methods of application tested the absorption ratio is lower. The usual rapidity of absorption from the cocoa butter preparations seems to rule out any significant interference by bacterial action. Further trials with different menstrua and improvement in the manufacture of suppositories it is hoped will reveal an even greater efficiency of penicillin absorption from the rectum and make it a useful means of systemic administration.

## SUMMARY

The absorption ratio of penicillin from the rectum is chiefly determined by the method of administration. It is very low after microclysters of penicillin solution, about 6 per cent in the case of cocoa butter suppositories, and subject to wide variations with gelatin capsules. Absorption of penicillin in insufflated or applied in cocoa butter capsules into the rectum is equivalent to upper intestinal absorption. The clinical use of the rectal route seems to depend upon ultimate selection of the most suitable vehicle.

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## PARENTERAL NUTRITION

### III STUDIES ON THE TOLERANCE OF DOGS TO INTRAVENOUS ADMINISTRATION OF FAT EMULSIONS

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PREVIOUS work in this laboratory has dealt with the problem of fat emulsions suitable for intravenous administration<sup>1,3</sup>. The desirability of fat as a part of parenteral nutrition is evident as a means of securing a high caloric intake with a minimum of fluid volume. With a sufficiently high caloric intake the utilization of protein from parenteral sources would be maximal, the destruction of body protein as a result of a caloric deficit would be minimal and it ought to be possible to improve considerably the nutritional status of a severely emaciated child or adult. That emulsions given intravenously are utilized has been shown in the dog by gain in weight by total carcass analysis for fat and by change from negative to positive nitrogen balance by increasing only calories as a result of intravenous fat<sup>3</sup>.

In the studies previously reported, a 15 per cent fat emulsion was infused into dogs at a slow rate. Granulomatous lesions were found principally in the lungs and spleen and to a less extent in the liver after daily infusion for thirty to ninety days. The fat emulsion contained in addition to fat a soybean phosphatide preparation as a stabilizing agent; this material may have been primarily responsible for these histopathologic lesions. The purpose of the present study is to report observations on the following: the tolerance of dogs to emulsions of 30 per cent fat and to the rapid administration intravenously of such emulsions; the length of time infused fat remains in the blood and whether the fat or the stabilizer is primarily responsible for the granulomatous lesions.

#### EXPERIMENTAL

A total of sixteen adult mongrel dogs was used in these studies. The fat emulsions were infused into the leg veins as usual. The preparation of the fat emulsions was similar to that previously used and the composition is given in Table I. Total plasma fatty acids were determined by the method of Bloor. The hematologic studies were made by the usual techniques, and the determinations of biomsulfalein elimination, plasma phosphatase and plasma cholesterol components were as previously described<sup>5</sup>. In spite of an epidemic of distemper which developed while the experiments were in progress the fat infusions were

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weight loss occurred during this second period of fat infusion which was similar to the first period in the emulsion used, the total amount of fat given, the rate at which it was given, and the number of days the animal was infused. Indeed, there was a gain in weight of 1.4 kilograms. The dog appeared perfectly normal at the end of the experiment. Bromsulfalein tests done periodically throughout the complete experiment (sixty-six days, see Fig. 1) suggested at times a moderate impairment of liver function as previously noted,<sup>3</sup> but results frequently were normal.

At post-mortem examination this animal (Dog 50) showed granulomatous lesions in the lungs which were similar to those described previously.<sup>2, 3</sup> These consisted of small nodules of two or three giant cells surrounded by round cells. There were also several larger collections of round cells and polymorphonuclear leucocytes that were not associated with giant cells. Small collections of macrophages with yellow pigment were in the alveoli. Large collections of polymorphonuclear leucocytes were seen in the portal areas of the liver but without granulomatous lesions or fatty metamorphosis. There were collections of round cells in the kidney and several large nodules of lymphoid and plasma cells and fibroblasts. No giant cells were observed. The heart, spleen, gastrointestinal tract, pancreas, adrenals, lymph nodes, blood vessels, and brain were normal.

Sections of the lungs of the other dogs of Experiment 1 that were alive at the completion of the experiment showed approximately four relatively small granulomatous lesions per low-power field. The livers of Dogs 52 and 48 showed polymorphonuclear leucocytes in the portal areas. There was no fatty metamorphosis of the liver in any of the animals. There was yellowish-brown pigment in the spleen and lymph nodes. The other organs were normal.

*Experiment 2*—The object of this experiment was to determine whether the fat or the phosphatide was principally responsible for the granulomatous lesions that had been observed following infusion of fat emulsions. In this experiment a total of six dogs was used. They were divided into three groups of two each and for the first two weeks of the study were fed horse meat and dog chow ad libitum though food consumption records were kept. During the next two weeks the amount of horse meat and dog chow fed daily was arbitrarily cut to half the average daily amount of the first two-week period. At the beginning of the third period (twenty-ninth day of experiment) all the dogs received intravenous infusions in addition to the amount of horse meat and dog chow given in the second period with the reduced rations. One group received Emulsion 11M (15 per cent fat), the second group, Emulsion 13 (2 per cent fat), and the third group, Emulsion 14 (no added fat). These infusions were given daily for the next thirty-two days in the amount of 12 to 14 ml per kilogram body weight per day and in a period of twenty minutes. The experiment was terminated on the sixty-first day for one of the dogs receiving the 15 per cent emulsion and for both of the dogs receiving the 2 per cent emulsion. The remaining three animals were infused for forty more days, though the two dogs that had received Emulsion 14 without any added fat were permitted ad libitum oral feeding. Since the response in weight for the animals in each group was similar, only one weight curve for each group is given, these are in Fig. 2. Hematologic and chemical data obtained on these dogs are given in Table III.

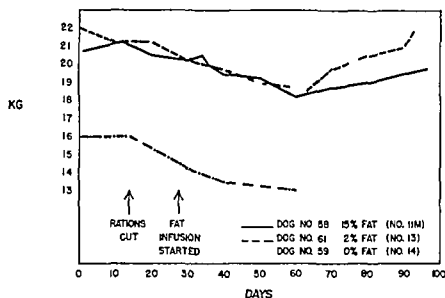


Fig —Weight curves of representative dogs in Experiment

## E III HEMATOLOGIC AND CHEMICAL DATA OBTAINED ON DOGS IN EXPERIMENT 2

Experiment	Dog 58							Dog 60						
	1	3	5	7	9	10	13	1	3	5	7	9	10	13
ion 11M given ated by bar														
1m per 100 c.c.)	13.5	13.5	11.8		12.0		10.9	15.1	14.4	13.4		13.1	12.7	
o)	40.6	38.9	34.5		35.9		32.8	40.1	40.9	40.5		38.3	38.1	
(%)	0.5	0.0	0.0		0.0		0.0	0.2	0.8	0.0		0.0	0.2	
test	6.0	6.0	11		12.0		8.0	10	10	12		10	12	
cholesterol		61	128		126		100	123	129	142		140	153	
erol esters		42	86		70		72	85	88	56		75	89	
fatty acids		452	453		69		400		222	393		310	347	
atase		55	100		150		195		169	190		300	354	
Experiment	Dog 61							Dog 64						
	1	3	5	7	9	10	13	1	3	5	7	9	10	13
ion 13 given IV by bar														
1m per 100 c.c.)	14.0	14.5	14.0		14.4			14.8	16.6	16.6	14.2	13.3		
o)	39.9	44	43.8		43.6			44.3	48	48.6	42.3	40.8		
(%)	0.1	0.0	0.2		0.0			0.0	0.2	0.8	0.0	0.0		
test		4	8		6			10	10	12	10	12		
cholesterol		153	236		179			130	107	182	200	173		
erol esters		103	167		106			85	69	91	110	115		
fatty acids		222	189		179			260	303	318	180	302		
atase		150	121		333			37	67	204	363	350		
Experiment	Dog 59							Dog 63						
	1	3	5	7	9	10	13	1	3	5	7	9	10	13
hosphatides 4) given IV as bar														
1m per 100 c.c.)	16.2	17.8	17.5	16.5	13.5	14.1	13.8	14.5	14.5	13.5	14.7		13.4	14.2
o)	53.6	49.2	50.3	49.2	40.0	43.1	41.1	45.5	45.1	40.0	43.4		43.6	47.2
(%)	0.0	0.1	0.0	0.0	0.4		0.0	0.2	0.0	0.0	0.0		0.3	0.0
test	12	10	10	8	12	10	8		6	4	4		7	10
cholesterol	131	163	149	160	175	197	146		110	100	77		130	140
erol esters	72	100	50	75	90	66	80		90	69	35		72	80
fatty acids		460	296	340	300	203	404		440	172	423		320	325
atase		90	162	200	175	154	355							

hosphatase micrograms of inorganic phosphorus liberated per cubic centimeter of plasma  
hours at pH 7.6

alein test micrograms of dye per cubic centimeter of plasma at eight minutes

otal cholesterol milligrams per cent.

holesterol esters milligrams per cent.

sma fatty acids milligrams per cent



**Fat tolerance curves** At various times throughout the fat infusion periods of the two dogs receiving the 15 per cent fat emulsion (Dogs 58 and 50) in Experiment 2, a number of fat tolerance studies were made. These consisted of determining the total plasma fatty acids immediately before the infusion and at varying intervals during and after. Two typical curves are shown in Fig 3.

**Post-mortem examination** All the dogs of Experiment 2 were autopsied at the termination of the experiment. The dogs that received either the 15 per cent or the 2 per cent fat emulsion showed essentially similar pathologic changes.

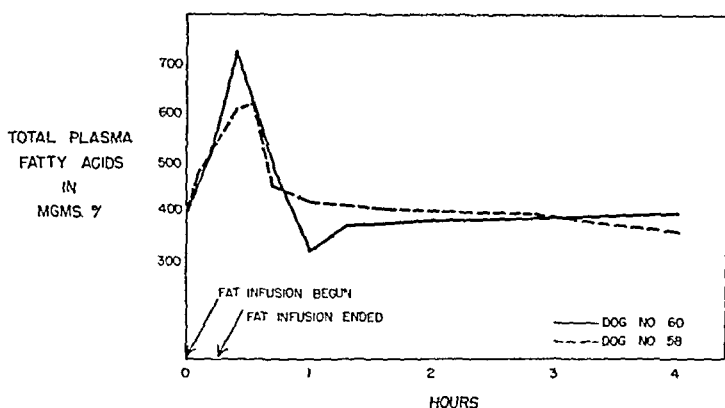


Fig 3—Fat tolerance curves in dogs following infusion of 15 per cent fat Emulsion 11 M in Experiment 2

Granulomatous lesions in the lungs were fewer and less severe than those found in the lungs of the animals in Experiment 1 and also less severe and less numerous than those found in any of the animals which we have previously studied. All of the four animals had evidence of distemper. No granulomatous lesions were found in any of the other organs.

Dogs 59 and 63 given the soy bean phosphatide alone, are of particular interest. More severe and more numerous granulomatous lesions were found in the lungs of these dogs. Foreign body giant cells were strikingly prominent in these lesions and particulate matter was identified in many of these cells.

**Experiment 3**—In this experiment a pregnant dog was given daily fat infusions. The dog was fed ad libitum a purified low fat ration (compare with ration 2, reference 3) during the first two week period. Food consumption records were kept and during the second two week period half the average daily consumption of the first period was given. At the beginning of the third period (twenty-ninth day of the experiment) daily fat infusions with Emulsion 11M (15 per cent fat) were started in addition to continuing the purified oral ration in the amount used in the preceding two week period. An arbitrary amount of 300 ml of fat emulsion was given daily. This furnished 460 calories, or an average of 33 calories per kilo of body weight. The quantity of purified ration fed amounted to 80 Gm per day and furnished 309 calories, or an average of 22 per kilo of body weight. The fat infusions and purified ration were continued daily until the day the pups were born. They were not given on the day of delivery.

on the day following but then were given as previously for a further period of thirty five days after which they were discontinued and ad libitum feeding of dog chow and horse meat continued for the rest of the experiment. On the day of delivery, milk, meat, and dog chow were fed ad libitum. A lobectomy was

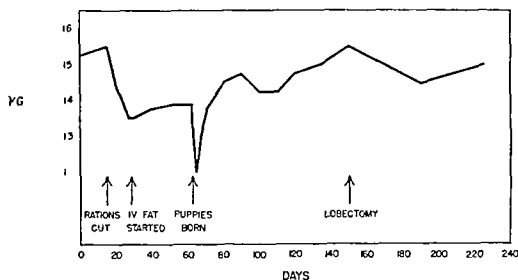


Fig 4—Weight curve for Dog 6 Experiment 3 fat infusion during pregnancy

done on this animal eighty eight days after delivery which was fifty two days after the last fat infusion. Weight and hematologic records were kept for an additional seventy five days after which the animal was sacrificed for post mortem examinations. The weight curve of this animal is given in Fig 4 and the chemical and hematologic data in Table IV.

TABLE IV HEMATOLOGIC AND CHEMICAL DATA OBTAINED ON DOG 56 IN EXPERIMENT 3, FAT INFUSION DURING PREGNANCY

Week of experiment	1	3	4	6	7	8	11	19	25	31
15% Fat emulsion 11M given IV as indicated by bar								†		
Hemoglobin (Gm per 100 cc)	15.6	14.9	14.0	11.6	9.7	9.7	9.4	13.1	15.9	16.1
Hematocrit (%)	50.7	48.7	42.2	32.2	29.8	29.9	9.9	9.6	51.8	49.2
Reticulocytes (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
Bromsulphalein	16	23		13		26	12			
Plasma total cholesterol	100	93		241		416	116			
Plasma cholesterol esters	73	80		124		160	69			
Total plasma fatty acids		384		309		518	584			
Plasma phosphatase		84		106		463	251			

\*Two pups born

†Lobectomy

Plasma phosphatase micrograms of inorganic phosphorus liberated per cubic centimeter of plasma in twenty four hours at pH 7.6

Bromsulphalein test micrograms of dye per cubic centimeter of plasma at eight minutes

Plasma total cholesterol milligrams per cent

Plasma cholesterol esters milligrams per cent

Total plasma fatty acids milligrams per cent

Post-mortem examination of this dog revealed no unusual findings grossly. Microscopic examination of the lobe of the lung removed surgically showed approximately one granulomatous lesion per two low power fields. The lesions were smaller and less numerous than those generally found. The lungs at autopsy showed even fewer granulomatous lesions, approximately one per ten low power fields. These lesions were even smaller than those seen in the lobe removed sur-

gically and there was very little scar formation. There was a fairly large area in the myocardium infiltrated with round cells and polymorphonuclear leucocytes. The liver showed focal areas of necrosis in the portal areas but no granulomatous lesions or fatty metamorphosis. There was hyperplasia of both the red and white series of the bone marrow. The spleen, gastrointestinal tract, pancreas, adrenal, lymph nodes, blood vessels, and brain were normal.

The autopsy on this dog was performed seventy-five days following the surgical removal of the lung and one hundred twenty-seven days after the last injection of fat. It is apparent from these findings that the lesions described tended to regress slowly, for there was a striking difference in the number and size of the lesions in the lung removed surgically and the lungs examined at the time of autopsy.

#### DISCUSSION

The studies in Experiment 1 demonstrate that fat can be given in an emulsion at least twice as concentrated as we had previously used (30 per cent as compared with 15 per cent) and at a much more rapid rate. These observations are of importance in the potential clinical use of fat emulsions because they indicate that a considerable number of calories in a limited fluid volume may be given in a relatively short time, thus eliminating a considerable increase in blood volume and prolonged periods of intravenous infusion.

The 30 per cent fat emulsion furnishes approximately 30 calories per milliliter. The dogs tolerated the emulsion well except for the first two or three days of the infusion period when there was considerable vomiting, but even this generally could be controlled if the emulsion was given slowly, especially during the first half of the infusion period.

Dog 50 of Experiment 1 is of much interest because it had two periods of fat infusions, the total amount of fat being the same in each period and quite large, namely 770 grams. The first of these infusion periods began a few days after the dog became distemperous and for a time the infused fat constituted the sole source of calories because the dog was completely anorectic. During this period the dog rapidly lost weight, but probably less than would have been experienced had the animal not been receiving fat. During the second infusion period, identical to the first except that the dog had recovered from distemper, the fat was well tolerated, weight gain was rapid, and the dog was in excellent condition at the end of the experiment.

In Experiment 2 a preliminary attempt was made to find out whether the fat or the phosphatide used as the stabilizing agent was principally responsible for the granulomatous lesions that had always been observed following infusion. For this reason, infusions of a 15 per cent fat emulsion, a 2 per cent fat emulsion, and an emulsion containing no added fat but simply the phosphatide preparation were made. Two dogs were given each preparation for varying periods of time as indicated in Table III. The histopathology produced in all of these animals was essentially the same but was more accentuated in the two dogs receiving the phosphatide alone. It appears that the phosphatide preparation is the basic cause of the granulomatous lesions we have observed.

The hematologic and chemical data obtained in the six dogs in Experiment 2 (Table III) reveal a consistent decline in the hemoglobin and hematocrit throughout the course of the infusions. With the exception of Dog 63, which was one of the animals receiving only the phosphatides, this change was essentially the same in all animals and was not dependent on the amount of fat in the emulsion. In none of the dogs was there any significant reticulocytosis suggesting that the anemia was not hemolytic. Bromsulfalein elimination in all dogs and plasma total cholesterol and cholesterol esters varied from normal to values somewhat higher. Plasma phosphatase values showed a definite increase throughout the infusion periods in all dogs except Dog 63.

Total plasma fatty acids values in the dog generally range from 200 to 450 mg per cent, depending principally on the diet and the time interval after eating that blood is taken for analysis. As the fat tolerance curves in Fig 3 show, there is a rapid rise in plasma fat following infusion of a fat emulsion to a value approximately twice the normal. As soon as the infusion is finished, the plasma fat begins to decrease and in the short time of approximately one hour is back to normal. It is evident that infused fat leaves the blood stream rapidly.

While Experiment 3 consists of observations on only one dog, it is of particular interest in that it presents the results of fat infusion in a dog throughout the last half of pregnancy. The average oral intake of this animal was decreased during the second two weeks of the experiment to one half the amount consumed during the first two week period. From Fig 3 it is seen that during the second two-week period there was a progressive weight loss totaling 2 kilograms. This weight loss was stopped at the end of the second two week period when infusions with the 15 per cent fat emulsion were started. These infusions furnished an additional 460 calories per day or 33 calories per kilo of body weight. The infusions were given daily beginning with the twenty ninth experimental day, except on the day of whelping and the day immediately following. They were discontinued on the ninety eighth experimental day. There were only two pups in the litter and they were small and scrawny, however both lived and developed into healthy dogs. The mother had no difficulty in feeding them.

A lobectomy was done fifty two days after the last of 120 daily fat infusions. The animal was sacrificed seventy four days following the lobectomy which was the two hundred and twenty fourth experimental day. At the end of the experiment the animal had completely made up all weight loss. Hemoglobin and hematocrit values at the end of the study were essentially the same as at the beginning, though throughout the course of the experiment both had fallen, due no doubt to the combined effects of pregnancy and the daily infusions. Bromsulfalein clearance, cholesterol values (both total and esterified), plasma fat and plasma phosphatase remained essentially normal except for the values obtained just prior to whelping when they were all increased as is shown in Fig 3.

#### SUMMARY

1. A 30 per cent fat emulsion stabilized with soybean phosphatides (Asolectin) was given intravenously to dogs with the same ease as the 15 per cent emulsions used in our previous studies.

2 Fat emulsions can be given relatively rapidly to adult dogs

3 Fat infused into the blood stream caused a prompt and marked increase in plasma fat but normal values were approached in the adult dog within an hour after the termination of the infusion

4 It appears that the soybean phosphatide used as the stabilizer was primarily responsible for the granulomatous lesions we have observed following the use of intravenous fat emulsions

We desire to express our appreciation to Miss Virginia Kent and to Miss Mary Maloney for technical assistance

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## PARENTERAL NUTRITION

### IV IMPROVED TECHNIQUES FOR THE PREPARATION OF FAT EMULSIONS FOR INTRAVENOUS NUTRITION

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IN PREVIOUS reports from this laboratory<sup>1, 2</sup> the use of fat emulsions for the intravenous nutrition of dogs has been described. One phase of the continuation of this problem has been the improvement of techniques for preparing emulsions of suitable particle size and stability. Since the relationship of particle size to such problems as fat embolism and fat utilization is not clearly understood at present, it seems desirable that *all* particles in such emulsions be no larger than normal chylomicra. When particles of this magnitude are dealt with, an accurate method of measuring particle size becomes of importance. In addition, there are the usual problems met with in intravenous nutrition that is the elimination of pyrogens, sterilization, and the prevention of pathologic changes resulting from the administration of the material under study. The present paper deals with a photomicrographic method for determining particle size, a method for preparing fat emulsions whose maximum particle size is under  $2\ \mu$  in diameter, and some of the preliminary studies on the physical stability of these emulsions.

#### EXPERIMENTAL

*Photomicrographic Method for Determining the Size of Fat Particles*—Quantitative measurements of fat particles below  $5\ \mu$  are difficult because of Brownian movement and low optical density. Since visual determinations are only approximate a photomicrographic method was developed. In preliminary work both a tungsten filament and a carbon arc lamp were used as light sources but were not found satisfactory at high shutter speeds. Subsequently a high speed high intensity, discharge lamp was found to be adequate. The apparatus is shown in Fig. 1. An Eastman precision enlarger No. 1 with a 35 mm. film roll adapter and holder was used without a shutter or lens but was provided with a bellows adapter for the microscope. A Spencer research model microscope with an apochromatic lens system was used. Eastman Kodak Plus X'' 35 mm. roll film is satisfactory and considerably more convenient than Kodak M plates of Type B panchromatic sensitivity.

The light source consisted of an Edgeton type, high voltage discharge lamp housed in a Spencer model 370 microscope lamp\*. The lamp had a discharge

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Monarch Electrical Company, Lynn, Mass.

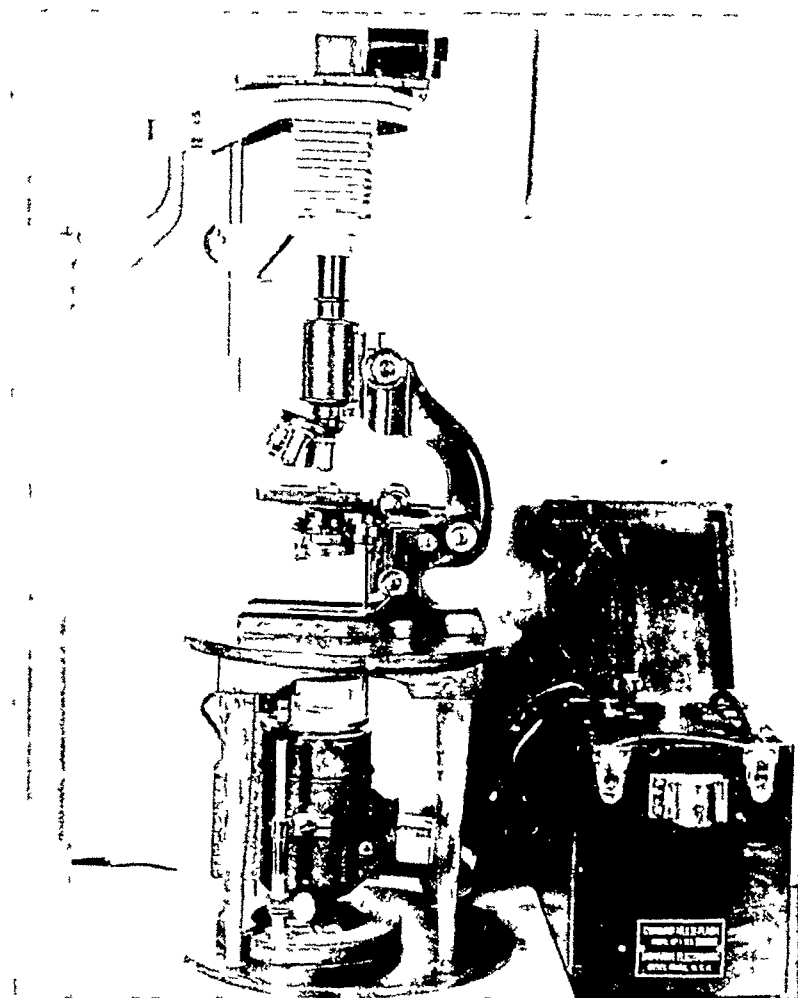


Fig 1—A photograph of the equipment used in preparing photomicrographs of emulsions

time of  $\frac{1}{50,000}$  second. Focusing of the microscope was accomplished by means of a General Electric 100 watt projection type tungsten filament bulb contained in the aluminum holder shown in Fig 1. The focusing lamp assembly was previously centered with respect to the optical axis of the flash lamp. After focusing, the holder was removed from its position atop the discharge lamp casing.

In practice, a thin sample of undiluted emulsion was placed between a slide and cover slip and the chamber sealed with paraffin to prevent evaporation. For examination of a preparation the sample was removed from the surface, since the largest particles in the preparation tend to accumulate there. For some purposes sampling with a pipette and bulb at varying depths from the surface was done.

Calibration was accomplished by photographing and enlarging a slide micrometer in the same manner as was used for the emulsions. Measurements were then made by means of rule and caliper.

By a series of trials it was possible to establish the proper lamp diaphragm, field stop, and bellows length settings which would produce optimum results. Since the intensity and duration of the illuminating source were constant, the field stop on the lamp was used to control intensity. The substage condenser diaphragm was used to develop contrast. In general, maximal contrast was developed with the condenser diaphragm aperture reduced considerably below the numerical aperture rating of the objective used.

The combination of lens systems used was determined by the dimensional range of the material to be measured. For the emulsion particles under  $2\ \mu$  the 2 mm objective with oil immersions above and below the slide and with a 10X ocular gave the best results. A Wratten B filter was interposed between the ocular and the camera as a means of further accentuating contrast. This filter transmits between 480 and 620 millimicron. The bellows length was kept short. Additional magnification was accomplished when necessary by enlargement of the negatives.

For measurement, 3 by 5 inch enlargements of the negatives were satisfactory. Development of the negatives was designed to produce maximal contrast, and prints were made on No. 4 (Eastman) high contrast paper.

In practice each preparation measured is searched under high dry magnification for fields showing the largest particles and several of these are photographed, because maximal particle size is of prime importance. As mentioned below, in these emulsions the vast majority of the fat particles are less than  $0.2$  to  $0.5\ \mu$  in diameter and thus beyond the resolution of the light microscope.

Fig. 2 illustrates representative photographs of three emulsions and the scale photographs used in the measurements.

*Preparation of Emulsions*—Several different methods of preparing fat emulsions were studied under similar conditions to determine which warranted extensive consideration. Emulsions were produced by means of (1) a Waring blender (2) a hand homogenizer (3) an ultrasonic generator\* and (4) a high pressure homogenizer†. Aqueous dilution of a nonaqueous solution of fat and stabilizer was also tested. In each case the following constituents were used: coconut oil, soybean phosphatides‡ and distilled water. Since the high pressure homogenization method yielded the best emulsion from the standpoint of particle size further studies were conducted using this method of production.

After preliminary work the following general procedure was adopted for carrying out an emulsification.

*Premixing of Emulsion Constituents*—Double distilled water previously boiled for four minutes was placed in a Waring blender with the fractionated phosphatide preparation§. The mixture was spun at top speed for two min.

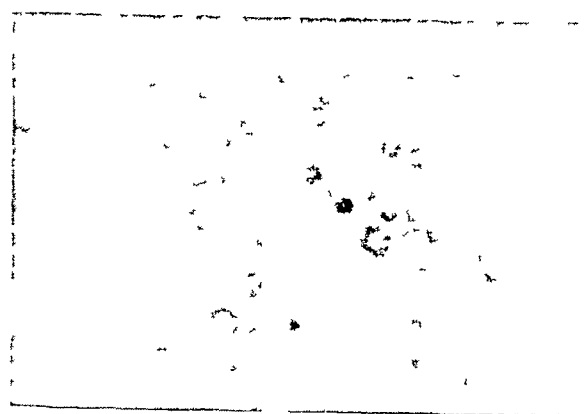
\*We are grateful to Dr. Ralph F. Shropshire and Mr. Edward W. Smith of the Submarine Signal Corp., Boston, Mass., for the use of their apparatus and for their assistance and cooperation.

†Junior Viscolizer 50, Cherry Burrell Corporation, Charlestown, Mass.

‡Isolectin supplied through the courtesy of Dr. Albert Scharf, Associated Concentrates Inc., Plmhurst, Long Island, N. Y.

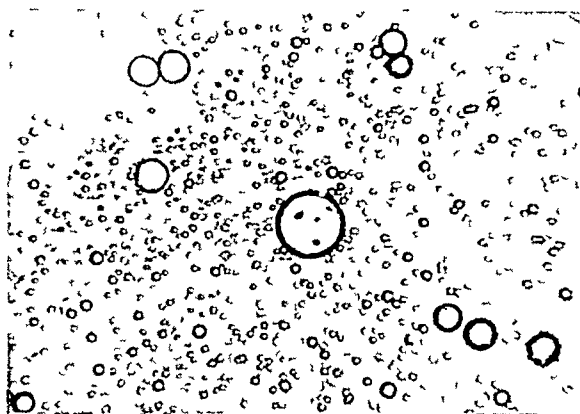
§This was prepared from commercial soybean phosphatides according to the procedure given in the following paper.





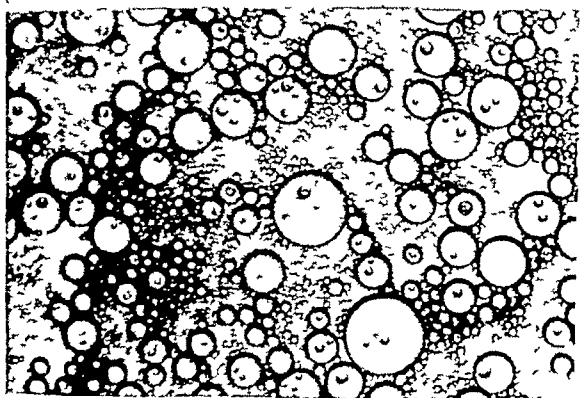
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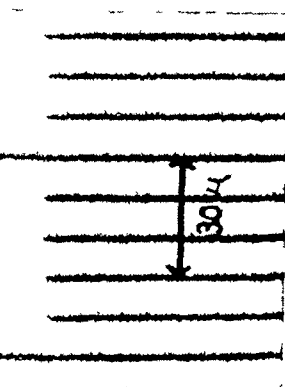
10 X

44 X

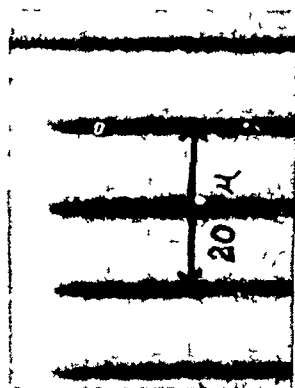


OCULAR 10 X

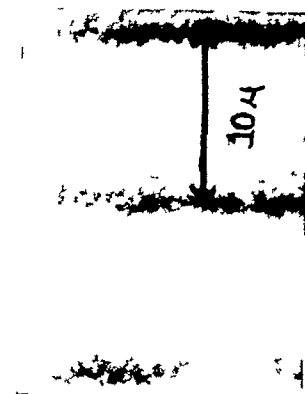
OBJECTIVE 20 X



A



B



C

Fig. 2.—Photomicrographs of fat emulsions and micrometer scales for measurement purposes. The three emulsions are shown in A, B, and C. The micrometer scales are shown in D, E, and F. The scales are graduated in microns and millimeters.

utes and hot coconut oil (80 to 95° C) was slowly added while the blender was still rotating. Blending was continued for an additional three to five minutes. Throughout the entire process a flowing atmosphere of nitrogen was provided by means of a suitable attachment tube on the cover. Though the fat particles ranged from 1 to 15  $\mu$  in diameter, this degree of dispersion was sufficient temporarily to prevent excessive "oiling out" when the material was placed in the high pressure homogenizer.

**High Pressure Homogenization Step**—The premixed material was introduced into the operating high pressure homogenizer which was equipped with the external glass recirculating system shown in Fig 3. The homogenizer was a smaller model of the machine used by McKibbin and co workers and was better adapted for small volumes of material. Prior to the addition of the blended emulsion, the machine was freed of water by operating with the pressure regu-

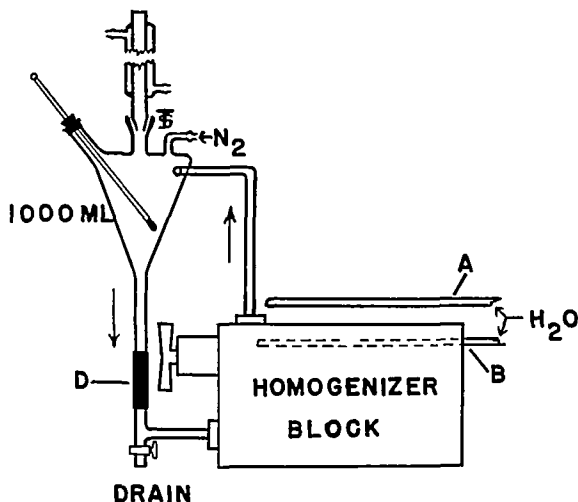


Fig 3—Diagram of high pressure homogenizing apparatus used in preparing fat emulsions for intravenous nutrition

lator block and the external intake port opened. By means of a long stiff wire bent in a right angle near one end the suction valves were lifted to allow the water in the middle chamber to drain. After all parts were again replaced and tightened the machine was run while nitrogen was supplied to displace the air. When the blended material had been added, the thermometer was put in place and the flow of nitrogen was adjusted to a very slow rate. Where required, heat was supplied by regulating the flow of hot water (76° C) through tube A (Fig 3). In those cases where a low temperature was desired the temperature of this water was lowered. In operation the condenser water flowed slowly

over the piston rods through an appropriately placed three-holed length of copper tubing (*B*). This made it unnecessary to use the cooling system provided by the manufacturer and insured better control of the temperature of the equipment and homogenate. To remove trapped gas, the machine was run at zero pressure and the rubber tube (*D*) was alternately closed and opened. If this was unsuccessful the pressure was raised to 2,200 pounds and maintained until the temperature of the circulating homogenate reached 75 to 78° C, at which point the gas either escaped or could be removed by closing the rubber tube.

Preliminary investigation demonstrated that although many particles below 1  $\mu$  were formed by single passage of the material through the homogenizer, some particles as large as 6 to 8  $\mu$  remained even after ten to fifteen passages at pressure between 2,000 and 4,000 pounds per square inch. For this reason the material was continuously recircled through the apparatus for periods of time ranging up to ninety minutes.

To determine the effect of temperature upon the rate of emulsification a series of tests were made using Emulsion 22 at 41, 60, 76, and 85° C. (For composition of Emulsion 22 see Table II.) The premixing in each case was done as described previously. It was found necessary to run cold water over the block to maintain the lowest temperature. Samples were removed at ten, twenty, and thirty-five minutes, and the size of the largest particles was ascertained. Table I contains the results of these tests carried out at a pressure of 3,000 pounds per square inch.

TABLE I EFFECT OF TEMPERATURE ON THE RATE OF EMULSIFICATION  
(VOLUME, 500 C C, PRESSURE, 3,000 POUNDS PER SQUARE INCH)

EXPERIMENT	TEMPERATURE (°C)	APPROXIMATE DIAMETER IN MICRA OF LARGEST PARTICLES OF A 30% FAT EMULSION (EMULSION 22)		
		10 MIN	20 MIN	35 MIN
1	41	8-10	4-6	<3
2	60	5-7	4-6	<3
3	76	4-6	2-4	<2
4	85	4-6	3	<1

The effect of pressure was studied by preparing 500 c c quantities of Emulsion 31 at 2000, 2500, 3000, 3500, 4000, and 4500 pounds per square inch respectively. (For composition of Emulsion 31 see Table II.) Homogenization was carried out for thirty to forty-five minutes at 76 to 80° C. Visual microscopic examination of samples removed at ten-minute intervals was used as a criterion. It was found that although all pressures used resulted in particles below 2  $\mu$ , the pressures above 3,000 pounds per square inch resulted in a greater proportion of the particles being at the extreme end of microscopic resolution. Thus emulsions prepared at higher pressures could be viewed microscopically when undiluted, without having the field of vision limited to the upper layers of the preparation. The emulsions appeared blue by reflected light in thin films and appeared red by transmitted light. These phenomena were observed with emulsions containing both 15 and 30 per cent of fat.

A number of emulsions were prepared in an investigation of the levels and ratios of fat and phosphatide conducive to small particle formation and stability. The amount of phosphatide was varied between 0.15 and 6.0 Gm per 100 cc, and the fat, between 10 and 30 Gm per 100 cubic centimeters. The fat was either coconut oil, corn oil or butter fat\*. The emulsions prepared and the data pertaining to the conditions of preparation are given in Table II. Also included are the size of the particles produced and the effect of autoclaving for fifteen minutes at 15 pounds per square inch.

Fat emulsions are adversely affected by high electrolyte concentration, low pH (below 6.0), prolonged heating, evaporation of the water phase, and the presence of materials which carry electric charges dissimilar to those of the fat particles. Many of the emulsions given in Table II were studied under these adverse conditions. These studies are still in progress and will be reported in full at a later date. It is clear that for a high fat level more emulsifier is required for stability under adverse conditions. In addition it has been found that, with a given level of emulsifier, as the concentration of oil increases the emulsions are formed with increasing difficulty. It also has been found that small particles are of prime importance to stability. Thus emulsions whose particles of fat are less than  $2\ \mu$  in diameter and have a probable mean diameter below  $0.5\ \mu$  are completely stable to autoclaving, whereas, otherwise comparable emulsions containing many particles above  $5\ \mu$  either break or cream out under the treatment or upon standing.

Emulsions similar to Emulsions 22 and 31 have been used for extensive animal investigation because they are stable and are compatible with blood both in vitro and in vivo studies. However as described in the following paper the soybean phosphatide preparation has been fractionated so as to give a more desirable stabilizing fraction, and this fraction (BF2) is used in place of the Asolectin. In routine practice the emulsions are autoclaved in gas tight bottles filled with nitrogen and with an aqueous dextrose concentration of 5 per cent. The bottles are stored in the dark at  $24^{\circ}\text{C}$  and before intravenous use 1 cc of sterile 10 per cent  $\text{Na}_2\text{HPO}_4$  is added to each 100 cc of emulsion. The pH of the emulsion is thus brought to 7.4. The results of the in vivo studies with these emulsions will be reported at a later date.

#### DISCUSSION

The photomicrographic technique has furnished a means of objectively evaluating emulsion preparations. It should be emphasized that the maximum particle size is the most important single factor in determining physical stability and may have more physiologic significance than simply assuring passage through the capillaries without embolization.

Under the proper conditions high pressure homogenization resulted in the formation of fat emulsions whose largest particles were below  $2\ \mu$  in diameter.

\*The butter fat was prepared by melting unsalted butter at  $65^{\circ}\text{C}$ , decanting the oil portion from the curd and water and shaking with anhydrous  $\text{Na}_2\text{SO}_4$  while warm. The fat was then filtered through a Buchner funnel using quantitative paper. The product was entirely clear. The butter was furnished through the courtesy of the H. P. Hood and Sons Co. Boston, Mass.

TABLE II COMPARATIVE STUDIES OF FAT EMULSIONS WITH REGARD TO TYPE OF FAT AND RATIO OF FAT TO PHOSPHATIDE EMULSIFIER

EMULSION	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Corn oil (Gm/100 cc)	-	-	10	20	30	30	30	-	-	-	10	-	-	-	-	-	-	-	-	-
Coconut oil (Gm/100 cc)	15	10	-	-	-	-	-	30	30	30	-	10	30	15	15	15	15	15	-	-
Butter fat (Gm/100 cc)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Asiolectin (Gm/100 cc)	27	20	20	40	60	0.5	30	30	30	0.4	30	60	60	0.1	0.15	0.15	30	0.15	15	30
N <sub>2</sub> HPO <sub>4</sub> (Gm/100 cc)	0.6	-	0.4	0.6	1.2	-	-	-	0.6	0.6	-	-	0.33	0.002	-	-	-	-	0.5	30
Dextrose (Gm/100 cc)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Volume (cc) × 100	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	3.5	5	5	5	4
Pressure (lbs/sq in) × 100	20	20	20	20	20	25.5	30	30	27	20	20	20	20	20	20	20	20	20	20	20
Time (min)	35	35	35	35	35	25	35	65	35	38	60	60	35	30	35	30	30	30	35	35
Diameter of particles (μ)	<2	<2	<3	<3	<3	<10	<2	<2	<2	<8	<2	<3	<2	<2	<2	<2	<2	<2	<4	<3
Effect of autoclaving*	N	N	N	N	N	B	N	N	N	C	N	N	L	L	N	N	N	N	N	N

\*N None B broke C creaming L some larger particles formed

Of primary importance was the continuous recirculation of the material through the machine for periods of time ranging from thirty to ninety minutes, depending upon the fat content. Shorter time intervals were inadequate and also negated the advantage of using high pressures. Thus, periods of five to ten minutes yielded no better emulsions at pressures above 3 000 pounds per square inch than were obtained at 2 000 pounds per square inch. Pressures above 3,000 pounds per square inch, however, when used for longer periods of time resulted in a shift in the mean particle size toward the lower limit of visual microscopy. Such emulsions took on a bluish appearance and transmitted red light even when the fat content was as high as 30 per cent. Light field microscopic examination of a thin layer of an undiluted 15 or 30 per cent fat emulsion revealed relatively few discernible particles. Dark field examination disclosed many additional particles below  $0.5 \mu$  in diameter. These characteristics indicated that most of the emulsion was a true colloidal suspension of fat in water.

The data in Table I show that high temperature was conducive to better and more rapid emulsification. When the homogenization was carried out at pressures above 3 000 pounds per square inch, the temperature rose to the boiling point provided no cooling water was used. This circumstance was helpful for both a high pressure and a high temperature were obtained simultaneously. Experience has shown that boiling may cause the emulsion to break to a certain extent probably due to loss of the water phase by surface evaporation. The beneficial effect of higher temperatures is believed to be due to lowered viscosity which permits a smaller valve opening at any given pressure, lowered interfacial tension and a decrease in the force necessary to shear the fat globules.

The levels of emulsifier and fat and the ratio between them were also found to be of great importance. In general the lower the fat level the shorter the length of time necessary for complete emulsification and the more stable the finished emulsion to high electrolyte concentration and to autoclaving and storage. Thus, a 15 per cent coconut oil 3 per cent phosphatide emulsion (Emulsion 31) was prepared in thirty minutes and was unaffected by autoclaving. Emulsion 22 which contained 30 per cent coconut oil and 3 per cent phosphatide required sixty five minutes of homogenization but was stable to autoclaving. Raising the level of stabilizer to 6 per cent made little difference in the homogenizing time required to produce a good emulsion and one stable to autoclaving. Emulsions containing 0.15 per cent phosphatide were stable to autoclaving when the fat concentration was below 15 per cent. Such emulsions, however, were broken by low electrolyte concentrations.

Added dextrose has proved satisfactory as a means of rendering the emulsions compatible with blood from the standpoint of tonicity and does not adversely affect the emulsion during autoclaving. Dextrose has the added advantage of contributing to the total caloric content. Extensive animal testing has been conducted using an emulsion similar to Emulsion 22. The composition and caloric content of Emulsion 22 is given in Table III.

TABLE III COMPOSITION AND CALORIC CONTENT OF FAT EMULSION 22

COMPONENTS	GRAMS	CALORIES
Oil	300	2700
Dextrose	35	140
Phosphatide emulsifier	50	210 (approx)
Water	634	-
Total		3050

Studies are in progress on the chemical and physical stabilities of these emulsions and on their pyrogenic properties. Also being investigated are possible costabilizers and other emulsifiers. A serious criticism of many surface active substances studied is the tendency to *in vitro* hemolysis even in the low concentrations necessary. The use of filtration through bacterial filters is being studied. It has been found that 30 per cent fat emulsions can be passed through filtering candles or pads without adversely affecting stability.

The use of high frequency sound waves as a means of emulsification has been studied by other workers.<sup>5</sup> The magnetostriuctive principle is used extensively in the dairy industry in the production of homogenized milk. Our studies with sonation were not extensive but using this procedure we were unable to make emulsions which were as satisfactory for our purpose as emulsions made by a pressure homogenizer. This failure to produce good emulsions by sonation seemed to be related to two factors: first, the material could not be circulated adequately through the effective field of high-intensity cavitation, and second, the intensity of the mechanical activity of the vibrating membrane was so great that appreciable amounts of metal were dispersed into the solution.

#### SUMMARY

A photomicrographic apparatus and method have been developed to allow accurate determination of the size of fat particles in emulsions.

By means of high-pressure homogenization fat emulsions were prepared in which all particles were below  $2\ \mu$  in diameter and most were beyond the resolving power of the light microscope.

Factors conducive to the preparation of such emulsions were high pressure, high temperature, continuous recirculation of the material being homogenized, and the proper fat-stabilizer ratio. Data on each of these are given.

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## PARENTERAL NUTRITION

### V STUDIES ON SOYBEAN PHOSPHATIDES AS EMULSIFIERS FOR INTRAVENOUS FAT EMULSIONS

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THIS laboratory has carried on research in an effort to develop fat emulsions suitable for parenteral nutrition<sup>1,4</sup> Previous reports have shown that fat emulsions given intravenously to dogs can be utilized as a source of energy, that dogs are able to handle large quantities of emulsified fat administered daily for long periods of time that the fat can be given in a concentration as high as 30 per cent and at a rapid rate and that the infused fat leaves the blood in a few hours However, in all the animals previously studied granulomatous lesions and scarring of the lungs, liver and spleen were noted in varying degree Preliminary observations pointed to the phosphatide preparation used as an emulsifier as the principal agent responsible for these lesions<sup>4</sup>

Since the lesions were produced by a soybean phosphatide preparation (Asolectin)\* alone and since this emulsifier appeared to be the best of a large number studied it was desirable to investigate it further In all of the previous studies on this problem adult dogs had been used as the experimental animal It would be advantageous to use a smaller laboratory animal for much of this work The purpose of this paper is to report a chemical fractionation of the soybean phosphatide preparation which yields an emulsifying agent which will not give rise to the histopathology previously observed and to report observations on an improved fat emulsion given intravenously to adult rats and to puppies

### EXPERIMENTAL

The plan of the first part of this study was to utilize the rat as an experimental animal to assay various fractions of the phosphatide preparation both alone and as part of the fat emulsion for any lesion producing properties Both Sherman and Hisaw strains of the albino rat were used The animals weighed from 150 to 250 grams and were approximately 3 months old In most experiments a group of six rats plus controls were used Each experimental animal was given a daily injection of the material to be tested for six successive days The animals were injected through a tail vein while under light ether anesthesia The volume injected varied from 15 to 25 ml but in all cases amounted to 1 ml per 100 Gm of body weight It was given in

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thirty seconds or less. Six days following the last injection (unless otherwise noted) the animals were sacrificed by etherization and bleeding. Sections of all organs were fixed in 10 per cent formalin solution, embedded in paraffin, and stained with hematoxylin-eosin. Frozen sections of formalin-fixed materials were stained for fat with Sudan III when indicated.

The various fractions studied consisted of the so-called purified soybean phosphatide preparation (Asolectin) used in our previous studies and derivatives of this material obtained by fractionation procedures. These were observed for lesion-producing properties when injected alone and as a component of fat emulsions. The materials were prepared for intravenous ad-

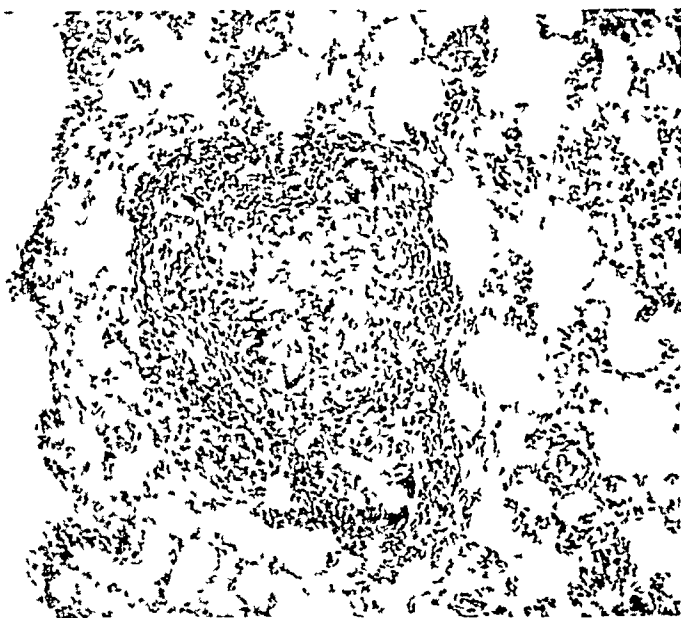


Fig. 1—Most severe type of granulomatous lesion. Note numerous giant cells, necrosis and polymorphonuclear leukocytic infiltration. Hematoxylin and eosin stain.  $\times 200$ .

ministration using one of the following procedures. (1) Homogenized preparations were made by the method recently described for the preparation of fat emulsions.<sup>\*</sup> The material was added to boiling water and spun for three minutes in a Waring blender at top speed. It was then homogenized in a high-pressure homogenizer<sup>†</sup> for thirty-five minutes. The pressure was maintained at 3,000 pounds per square inch, and the temperature, at 75 to 80° C. The process was carried out under a nitrogen atmosphere. Solutions were buffered with phosphate to a pH of 7.4 and made isotonic with either glucose or sodium chloride. The material was placed in brown glass pressure bottles, the air was displaced with nitrogen, and the tightly capped bottle was autoclaved at 15 pounds per square inch for fifteen minutes. (2) Blended preparations were obtained in the same manner except that the

<sup>\*</sup>Junior Viscolizer No. 50. Cherry Burrell Corporation, Chicago.

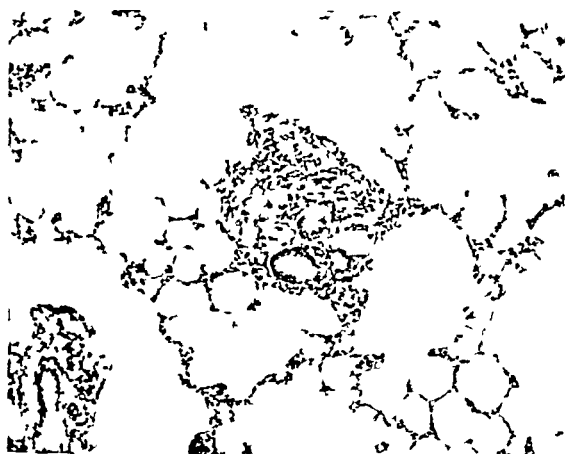


Fig. 2—Less severe lesions with few giant cells. Note absence of necrosis and small degree of fibrosis. Hematoxylin and eosin stain.  $\times 15$ .

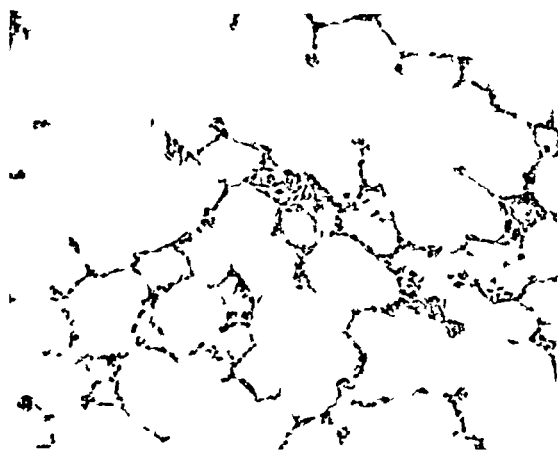


Fig. 3—Minimal lesion made up of one to two giant cells without accompanying necrosis or fibrosis. Hematoxylin and eosin stain.  $\times 300$ .

high-pressure homogenization was omitted (3) Crudely mixed preparations were made by adding the material to water (50° C) and spinning in the blender at high speed for thirty seconds. Tonicity and sterility were achieved as with the homogenized preparations.

In the experiments to be described the pathologic lesions were restricted to the lung and were not evident grossly. On microscopic examination the most severe lesions were granulomatous in type (Fig 1). The individual lesions consisted of groups of mononuclear cells and fibroblasts with one to four giant cells of the Langerhans type at the center. In the larger nodules Mallory's aniline blue stain demonstrated loose, fine, collagenous fibers arranged in concentric fashion. Seen among the fibers were large epithelioid cells. A few of the larger lesions had necrotic centers with many polymorphonuclear leucocytes. The giant cells contained from a few to twenty or thirty nuclei. These were arranged about the periphery of an abundant amount of light basophilic to eosinophilic cytoplasm. The cytoplasm was homogenous for the most part. However, some giant cells contained small droplets that stained red with Sudan III while in other giant cells particulate matter was seen. Refractile bodies were not observed when a frozen section of the tissue was examined through a polarizing microscope. Ziehl-Neelsen stain demonstrated no acid-fast material in the cytoplasm. No asteroid bodies were seen in the cytoplasm. The nuclei had prominent nuclear membranes and centrally placed nucleoli. The lesions in the more severe cases varied in size from 0.1 to 1.0 millimeter. In some instances the nodules were confluent. They had no constant relationship to bronchi or vessels. There was steady regression in the size of the lesions in animals which were allowed to live for a longer period than that stated.

The less severe lesions were seldom over 0.5 millimeter (Fig 2). There was little or no fibrosis and no necrosis. The nodules consisted of mononuclear cells and one to two giant cells, which were similar to those seen in the largest lesions.

Some of the lesions consisted of only one to two giant cells with no cellular elements or fibrosis about them (Fig 3). These were seen in the alveolar walls with only a separation of the basement membranes on each side.

The sections were graded according to the severity and frequency of the giant cell lesions. Four plus lesions were the most severe. In these there were three to four nodules per low-power field which were often confluent. The lesions themselves were large, with fibrosis and some necrosis. Three plus lesions were smaller with little fibrosis and no necrosis. However, they were almost as frequently seen as the four plus, occurring from one to three times per low-power field. Two plus lesions were small and infrequent, one lesion being observed per three high-power fields. One plus lesions consisted of rare isolated giant cells with no cellular components about them.

#### *Rat Assay Experiments, Part I —*

*Experiment 1* The soybean phosphatide preparation (Asolectin) in a concentration of 3 per cent in boiling water was blended in a Waring blender for a period of ten minutes. Microscopic examination of the material showed

that the suspended particles varied from 10 to 20  $\mu$  in size. Six adult rats were injected with this material. Sections of the lungs from all the assay animals showed numerous four plus lesions. In the center of some of the lesions there was definite necrosis and infiltration by polymorphonuclear leucocytes. These lesions were more numerous and larger than those found in any of the subsequent groups. These results and those of subsequent experiments are given in Table I.

TABLE I SUMMARY OF RAT ASSAY EXPERIMENTS ON INTRAVENOUS ADMINISTRATION OF PHOSPHATIDE PREPARATIONS AND FAT EMULSIONS

EXPERIMENT	MATERIAL GIVEN	CONCENTRATION (%)	MAXIMUM PARTICLE SIZE ( $\mu$ )	GRADING OF LESIONS					
				1	2	3	4	5	6
1	Blended commercial phosphatides	3	20	++++	+++	+++	++++	++++	++++
2	Homogenized commercial phosphatides	3	2	+	+	+	+	+	-
3	Homogenized commercial phosphatides	9	2	+	+	-			
4	Fraction A(F1)	3	2	+	+	+			
5	Fraction B(F1)	3	2	-	-	-			
6	Fraction C(F1)	3	2	+++	+++	+++			
7	Fraction A(F2)	0.09	5	+	+	-			
8	Fraction B(F2)	3	2	-	-	-			
9	Fraction B(F2)	3	5	-	+	+	-	-	-
10	Fraction A(F2)	0.09	14	+++	+++	+++			
	Fraction B(F2)	3	2						
	Fraction C(F2)	1.5	9	+	+	-			
	Fraction B(F2)	1.5	2						
11	Aspergillus niger	-	7	+++	+++	+++	+++	+++	+++
12	Emulsion of coconut oil & fraction B(F2)	0.0	2	-	-	-	-	-	+
		3							
Control group (12 animals)		-	-	-	-	-	-	-	-

Animal died one hour following last injection

*Experiment 2* The soybean phosphatide preparation (Asolectin) in a concentration of 3 per cent in water was homogenized. The particle size in this emulsion was 2 $\mu$  or smaller. A group of six rats were injected. Sections of the lungs from all animals showed small one plus lesions. There was little fibrosis or infiltration and no necrosis.

*Experiment 3* The soybean phosphatide (Asolectin) in a concentration of 9 per cent in water was homogenized. It was injected into three rats daily for only two days. The animals were sacrificed on the twelfth day of the experiment. Sections of the lungs were graded one plus and were similar to the lungs of the animals in Experiment 2. This experiment was carried out to determine if the total amount of phosphatide homogenate could be given in a shorter period of time without changing the number or severity of the lesions. It was concluded that, within limits, the rate of administration of the homogenized phosphatide was not a significant factor in the production of the lesions.

*Fractionation of the Phosphatide Preparation, Asolectin*—While the homogenized phosphatide preparations produced relatively few lesions it was felt that further experiments should be carried out to eliminate, if possible, the factor or factors responsible for the few lesions that were present without destroying any of the emulsifying properties of the phosphatides. Consequently, several chemical fractionation procedures were done on the purified soybean phosphatide and many of these preparations were then assayed for lesion-producing properties as in the previous experiments.

*Fractionation 1 (F1)* One hundred grams of soybean phosphatides were dissolved in 500 cc of chloroform. To this solution 1,500 cc of acetone were added slowly, with stirring, to yield a finely divided precipitate of phosphatides. After filtration the precipitate was redissolved in 100 cc chloroform and reprecipitated with 300 cc of acetone. After filtration the solid product was washed twice with acetone and was then freed of solvent by means of vacuum at room temperature. The combined filtrates from the preceding operations were taken to dryness under vacuum in a stream of nitrogen and yielded 18.2 Gm of a dark, gummy material which was given the designation A(F1). The acetone precipitate was added to 350 cc of absolute alcohol (63° C) and was spun in a Waring blender at high speed for three minutes. The suspension was cooled to room temperature and placed in the refrigerator overnight (7° C). After filtration through a Buchner funnel, the precipitate was washed with three 30 cc portions of cold absolute alcohol. The entire alcohol treatment was repeated except that the material was filtered after seven hours. Removal of the residual solvent from the precipitate yielded 41 Gm of a granular, light-tan material which was designated B(F1). The combined filtrates from the alcohol treatment were concentrated in vacuum under nitrogen and furnished 19.4 Gm of a light-yellow, amorphous substance. This was designated C(F1). The yields of both C(F1) and B(F1) were low because of loss during the initial step of this method.

*Fractionation 2 (F2)* It was found that a diethyl ether solution of soybean phosphatide remained turbid. This insoluble material would have appeared in fraction B(F1) of the previous procedure. Therefore, 50 Gm of phosphatides were dissolved in 100 cc of diethyl ether and the insoluble particulate material was removed by centrifugation. This material was washed five times with 10 cc portions of diethyl ether and was then freed of solvent under vacuum. This yielded 31 mg of a white powder which was designated A(F2). Microscopically this material comprised some particles which closely resembled rod-shaped bacteria and other particles which were of many irregular shapes and which ranged in size from less than 1 micron to 15 micra. Subsequent chemical determinations demonstrated that much of this material gives color tests characteristic of denatured proteins.

The filtrate from the preceding paragraph was made up to 200 cc with diethyl ether and was placed in the refrigerator (-7° C) for forty-eight hours. A slight cloudiness developed but disappeared on warming. Fifty cubic centimeters of acetone were added and after thorough shaking the solution was

placed at  $-7^{\circ}\text{C}$  overnight. The top layer was decanted and the bottom layer was carried through the diethyl ether acetone procedure again. After decantation, the top layers were combined and were concentrated under vacuum in a stream of nitrogen. The yield was 5.4 Gm of a very dark, viscous, gummy substance. This comprised preparation C(F2).

The bottom layer from the previous procedure was filtered through a Seitz filter using positive pressure. Concentration under vacuum yielded 32 Gm of a light yellow, granular material B(F2).

#### *Rat Assay Experiments Part II—*

*Experiment 4* A 3 per cent suspension of fraction A(F1) was prepared by mixing in a Waring blender for three minutes with boiling water. After autoclaving this material it was injected into six adult rats daily for a period of six days. The dosage was as previously mentioned for all of these experiments. The rats were sacrificed on the twelfth day of the experiment. Sections of the lungs showed one plus lesions.

*Experiment 5* A blended 3 per cent suspension of fraction C(F1) in water was injected into three rats daily for six days. The rats were sacrificed on the twelfth day of the experiment. Sections of the lungs were graded three plus for lesions. These lesions while as numerous as those found in the animals injected with blended whole phosphatide (Experiment 1) were not so large. Also, there was less inflammatory reaction and fibrosis and foreign body giant cells were less numerous per lesion.

*Experiment 7* A 9.5 mg per cent suspension of fraction A(F2) in water was injected into three rats daily for six days. This material was prepared for injection by grinding in a mortar with 5 Gm of dextrose and then was taken into water. The rats were sacrificed on the twelfth day of the experiment. Sections of the lungs were graded one plus for lesions.

*Experiment 8* Two groups of animals were used to test a blended preparation of fraction B(F2) in which the particle size varied from 1 to 2 micra. It was injected into six rats daily for six days. These rats were sacrificed on the twelfth experimental day. Sections of the lungs showed no lesions.

*Experiment 9* In the second assay with fraction B(F2) it was again made up in 3 per cent concentration in water but was crudely mixed in the Waring blender. The particle size varied from 1 micron to 8 micra. This material was injected into four rats daily for six days. The animals were sacrificed on the twelfth experimental day. No lesions were found in the lungs of two rats. The remaining two rats had lesions graded one plus. These lesions were small with no fibrosis or cellular infiltrates.

*Experiment 10* Fraction C(F2) was not suitable for injection by itself because of its high content of fatty acids and neutral fats and consequent insolubility in water. For this reason blended preparations were made up as follows: the first contained fractions A(F2) plus B(F2) and the second fractions C(F2) plus B(F2) both fractions in a concentration of 1.5 per cent in water. The B(F2) preparation was thus used as an emulsifier for the insoluble

A(F2) and C(F2), and the total concentration of each combined fraction was 3 per cent. Three rats were given six daily injections of fractions C(F2) plus B(F2) and were sacrificed on the twelfth experimental day. The lungs were graded one plus. Three additional rats were given six daily injections of A(F2) and B(F2) and were killed on the twelfth experiment day. The lungs were graded two plus in one rat and three plus in the other two. There were a few medium sized lesions but the majority were small.

*Experiment 11* It was felt that microorganisms growing in the crude products might be partially responsible for the production of lesions even though they were killed by autoclaving. The coconut oil, crude phosphatide, and water from the homogenizer were cultured. *Staphylococcus albus* and *Aspergillus niger* were recovered from the crude phosphatide. The fungus was grown in pure culture, suspended in isotonic saline autoclaved at 15 pounds pressure for fifteen minutes, and injected into six rats in a manner similar to that used in the other experiments. The rats were sacrificed on the twelfth day of the experiment. The lungs were graded three plus for lesions. Spores and hyphae were demonstrated in the giant cells. In this instance, lesions were also found in the spleen and liver.

*Experiment 12* An emulsion (Emulsion 35)<sup>5</sup> containing coconut oil, 300 Gm, phosphatide fraction B(F2), 30 Gm, dextrose, 35 Gm, and water, 634 ml, was prepared and injected daily for six days into a group of six rats. The particle size of this emulsion was less than 2  $\mu$ , predominantly less than 0.7 micron. The dosage was 0.5 cc per 100 Gm of body weight. The animals were sacrificed on the twelfth experimental day. Only one small lesion was found in one lung of the six rats examined.

*Control Animals for Rat Assay Experiments* A group of twelve rats served as controls. These animals were kept in the same cage as the experimental animals and were distributed throughout the twelve experiments. The lungs were examined and no lesions such as those described were present in any of the twelve control animals. An occasional rat in both the experimental and control groups showed pneumonitis such as is commonly encountered in laboratory rats. The lesions described herein were sufficiently characteristic to be distinguished from this type of pneumonitis.

#### *Puppy Experiments —*

*Experiment 13* A more extensive study of Emulsion 35 as prepared by the improved procedure described in the previous report<sup>5</sup> and using fraction B(F2) prepared from soybean phosphatides was carried out in puppies. Assay in rats had indicated that this phosphatide fraction would not lead to visceral lesions when infused as a water emulsion alone in a 3 per cent concentration or when used in that concentration as a component of a 30 per cent fat emulsion.

A group of four Labrador puppies, all littermates raised in this laboratory, was used. The animals were in apparent good health. They were wormed and during the course of the experiment were given canine distemper serum at two week intervals. Despite this precaution there were indications toward the end of the experiment that the animals were infected. Anorexia, fever, and leu

cocytosis suggested that a respiratory, bacterial infection was present. The animals were divided into two groups. The three animals in the first group were infused daily with amounts of emulsion varying from 15 to 10 Gm per kilo of body weight. The animals were fed a purified diet at the outset and were later transferred to a natural diet\*. The fourth animal served as a control and was not infused.

Immediate reactions to the intravenous infusion of the emulsion were for the most part determined by the rate of the infusion. This aspect of the work and detailed metabolic studies will be reported in a later paper. Pertinent to this discussion, however, was the observation that on several occasions various dogs became dyspneic at the start of the infusion. Expiration became difficult, the animal wheezed audibly, and there were forced attempts to clear material from the trachea. In each instance auscultation revealed profuse expiratory rhonchi and wheezes. Upon discontinuation of the infusion the animal appeared well within fifteen minutes and the abnormal signs had disappeared. Examination microscopically of the emulsion involved revealed loose aggregations of particles 2  $\mu$  or less in diameter which constituted a flocculus or raft like mass measuring up to 25  $\mu$  in diameter. The nature of the conditions leading to this phenomenon is not clear. Table II presents the data concerning the number of

TABLE II. SUMMARY OF INTRAVENOUS FAT INFUSION IN PUPPIES

PUPPY	DAYS INFUSED	TOTAL EMULSION INFUSED (CC)	TOTAL FAT INFUSED (GM)	TOTAL EMULSIFIER B(F2) (GM)	DAY OF EXPERIMENT SACRIFICED
904	0	0	0	0	52
907	30	2,343	703	70.3	49
905	42	4,704	1,411	141.1	56
909	84	10,295	3,089	308.9	84*

\*Animal died after prolonged massive doses of emulsion leading to anorexia

days the animals were infused and the amounts of emulsion and total fat infused. Puppy 904, the control animal received no infusions, Puppy 907 received 703 Gm of fat over a thirty day period, Puppy 905 received 1,411 Gm of fat over a forty two day period and Puppy 909 received 3,089 Gm of fat over an eighty four day period. All of these puppies were sacrificed for post mortem examination at varying times as indicated in Table II. Gross examination at autopsy revealed normal appearing organs in all puppies. Sections were made of lung, liver, spleen, kidney, brain, intestine, bone marrow, adrenal and lymph nodes and were stained with hematoxylin and eosin.

Two animals (Puppies 905 and 907) were sacrificed by etherization and bleeding. Both animals had pulmonary findings consistent with a mild distemper. The lungs from both animals also showed occasional giant cell lesions which were graded one plus. No granulomatous lesions such as were described previously were found in any of the other organs. No hemosiderin deposition was noted in the spleen, liver, lymph nodes, or bone marrow. The liver was stained with Sudan III and no fat was found. There was hyperplasia of the white cell series in the bone marrow.

Gaines Dog Meal furnished through the courtesy of the Research and Development Department, General Foods Corporation, Hoboken, N. J.



Puppy 909 died during the night and all organs were markedly autolyzed. As far as could be determined there were no granulomatous lesions present. There was pulmonary edema and many of the smaller bronchi were plugged with mucous.

The control animal (Puppy 904) also showed pulmonary lesions consistent with mild distemper. No other lesions of consequence were present in any of the organs.

#### DISCUSSION

In previous studies<sup>2,4</sup> it was shown that dogs receiving emulsions of corn oil or coconut oil in concentrations of 15 per cent, 30 per cent, or 2 per cent developed granulomatous lesions which were most marked in the lungs and more rarely found in the spleen, kidney, and liver. Essentially the same lesions were found when the emulsifying agent, a soybean phosphatide preparation, was given intravenously without added fat.<sup>4</sup> In the present report it is shown that such lesions were also produced in the lungs of the albino rat under similar conditions. The liver, spleen, kidney, heart, and brain of the rats were studied but no lesions were found. The rat proved a good experimental animal for assay of the emulsions and various phosphatide fractions because only small amounts of material were required for the development of the lesions. Thus only 30 mg of the original phosphatides per 100 Gm of rat per day for a six-day period were required to produce numerous lesions. That the assay time could be reduced further is shown in Experiment 2 in which 180 mg per 100 Gm of rat given for two days also produced typical lesions. Histologic examination of the lungs seemed to provide an accurate index of the lesion-producing properties of the test materials.

The larger lesions produced in the lung were made up of mononuclear cells, fibroblasts, and foreign body giant cells. Various substances were demonstrated in some of the giant cells. Some contained neutral fat, while in others particulate matter was observed. The lesions of the rats receiving the mold showed spores and hyphae in the giant cells. In other instances small bits of leather fragments were demonstrated. The latter probably came from the leather gaskets used in the homogenizer. Preliminary experiments have suggested that by using plastic gaskets and passing the finished emulsion through a suitable filter, particulate fragments can be completely removed.

The soybean phosphatide preparation (Asolectin) used in our previous studies was fractionated to yield an emulsifying agent more suitable for intravenous use. The first fractionation of the phosphatides was unsatisfactory because the small amount of ether-insoluble material present originally was not removed prior to subsequent steps. In the second fractionation this material, fraction A(F2), was removed at the start of the procedure and consisted of particles of solid material whose greatest dimension was as large as 12 micra. Such particles would be sufficiently large to block the alveolar capillaries in addition to setting up giant cell reactions. Significantly, this fraction gave color tests characteristic of denatured proteins. Since prolonged high pressure homogenization was fairly effective in preventing lesion formation, it

is probable that the particulate material was rendered small enough to escape being caught in the capillaries. This precludes the possibility of a chemical inactivation of the emulsive factor(s). The material which comprised fraction A(F2) would be present in fraction B(F1) of the first fractionation. The latter fraction caused extensive lesions. The material present in fraction C(F2) would consist of fat, fatty acids, sterols, lipid degradation products, waxes, and other substances soluble in an acetone ether mixture. From the standpoint of lesion production this fraction was of low potency. Its removal from the phosphatides is warranted, if for no other reason than its lack of emulsifying properties.

Fraction B(F2) should consist mainly of phospholipids and represents most of those originally present, for in practice an overall yield of 90 per cent is obtained. This fraction proved to be the most efficient for our purposes. In Experiment 8 a blended preparation with particle size varying from 1 to 2  $\mu$  was injected into six rats without producing lesions, in Experiment 12 it was injected as part of a 30 per cent fat emulsion (Emulsion 35) into six rats of which five were found free of lesions and the remaining animal showed only one small lesion in one lung. That this fraction is a good emulsifying agent is indicated by the fact that a 30 per cent coconut oil emulsion can be stabilized with 3 per cent of the fraction. Preparations of fraction B(F2) which have been kept at  $-7^{\circ}$  C for three months still retain their emulsifying properties. From Experiment 9 it can be seen that the injection of a crude mixture of fraction B(F2) in which the particles were 1 to 8  $\mu$  gave only a few one plus lesions. This suggests that within limits the particle size of these phospholipids is not of itself of great importance.

The twelve control rats for these assay experiments did not show the granulomatous lesions of the lung that had followed the injection of fractions other than B(F2).

The puppy experiments were of interest because they showed that fat could be given intravenously to this species without producing the extensive granulomatous lesions that had characterized all of our previous work. Also these studies were our first experiments with the administration of fat intravenously to growing animals. The fat emulsion used (Emulsion 35) contained 30 per cent coconut oil and was stabilized with a concentration of 3 per cent of fraction B(F2). The fat emulsion was well tolerated by the three puppies that received it depending largely on how rapidly it was given, particularly during the first few days of infusion. The growth and metabolic data of these puppies will form part of the substance of a subsequent report.

#### SUMMARY

A procedure to assay fat emulsions for intravenous use and the constituents of such emulsions has been developed using the albino rat.

Using this assay procedure confirmatory evidence was obtained that the soybean phosphatide material used as an emulsifying agent in our previous emulsions was the principal cause of the granulomatous lesions observed following the intravenous administration of fat emulsions.

Chemical fractionation of the soybean phosphatide preparation was accomplished so as to yield a fraction with good emulsifying properties and little or no lesion-producing properties

A 30 per cent fat emulsion (Emulsion 35) was given in daily intravenous infusions to three puppies in varying amounts and for periods of time varying from thirty to eighty-four days without producing the granulomatous lesions found in previous experiments with adult dogs

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## PARENTERAL NUTRITION

### VI FAT EMULSIONS FOR INTRAVENOUS NUTRITION THE TURBIDIMETRIC DETERMINATION OF INFUSED FAT IN BLOOD AFTER INTRAVENOUS ADMINISTRATION OF FAT EMULSIONS

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ONE phase of the research on the intravenous use of fat emulsions has been the determination of the rate of disappearance of the infused fat from the blood<sup>1</sup>. The usual methods for determining blood lipids are laborious and time consuming, and even the microadaptations of these methods require quantities of blood large enough to prohibit repeated use on small laboratory animals. Therefore the turbidimetric method described in this paper was developed. It has the advantage of requiring only 20 cmm of blood for a rapid accurate determination. Thus, numerous samples may be taken and the animals' ability to remove infused fat from the blood stream can be easily ascertained. The term "fat tolerance curve" is proposed for the graphic representation of the data obtained.

The present paper gives the method used, proof of its validity and the fat tolerance curves of the rat, dog, and rabbit when given 30 per cent fat emulsions.

#### EXPERIMENTAL

*Principle of the Method*—After the intravenous infusion of a fat emulsion the blood plasma becomes turbid. This turbidity is measured in a photoelectric colorimeter\* and the amount of infused fat is calculated from a standard curve or K value. Within limits the turbidity bears a straight line relationship with the instrument readings and fat concentration. By taking successive samples of blood after an infusion of fat, a fat tolerance curve can easily be obtained by plotting the turbidity readings against time.

#### *Materials Used*—

1 Thirty per cent coconut oil emulsion. The composition, preparation, sterilization, and determination of particle size of this emulsion (Emulsion 35) have been described previously.<sup>2</sup>

2 Five per cent dextrose solution

3 Concentrated ammonium hydroxide

4 Superoxol. Thirty per cent solution of hydrogen peroxide

*Preliminary Studies*—Originally the method used was to read the turbidity of a diluted sample of plasma in the photoelectric colorimeter using filter

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\* Klett Summer on photoelectric colorimeter was used in these studies.

No 66 This filter was chosen because the presence of small amounts of hemoglobin had little effect upon the readings. Investigation demonstrated that the turbidity read, however, was the summation of that of the fat and of extraneous turbidity caused by a reaction between the emulsion and plasma. This interfering turbidity could be removed by the addition of a small amount of ammonium hydroxide without influencing the fat emulsion. It was also found that the emulsion component responsible for the false turbidity reading lay in the phosphatide preparation used. Microscopic dark-field examination of a mixture of phosphatide and plasma revealed many nonspherical, highly refractile bodies which could be made to dissolve by touching the edge of the liquid under the cover glass with ammonium hydroxide. This phenomenon was not observed with either the phosphatide or the plasma alone. The following experiment was carried out to study quantitatively the reaction between the plasma and the phosphatide and also to determine whether the ammonium hydroxide influenced the fat emulsion. A 30 per cent fat emulsion was diluted with water and 4 ml samples were pipetted into standard Klett tubes. Various amounts of clear dog plasma were added and after thorough mixing the tubes were filled to the 5 ml mark with water. The turbidities were read and then to each tube was added 0.05 ml of concentrated ammonium hydroxide. After mixing, the turbidities were again read. The results of this experiment are shown in Fig 1.

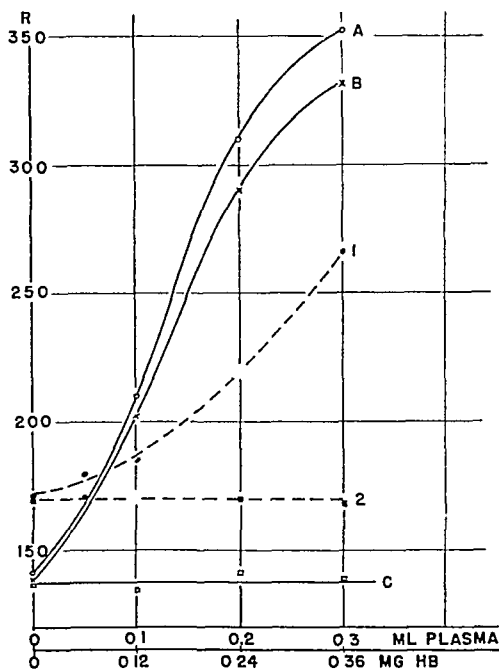


Fig 1

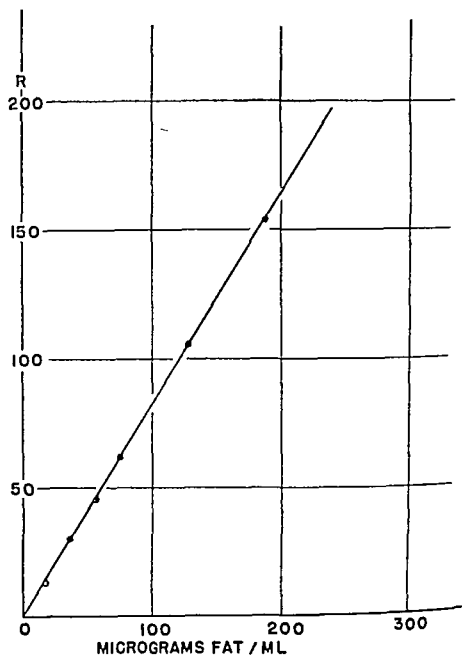


Fig 2

Fig 1—The effects of plasma and hemoglobin on the turbidity of fat emulsions. 1, Plasma plus emulsion; 2, plasma plus emulsion after  $\text{NH}_4\text{OH}$  treatment; A, hemoglobin plus emulsion; B, hemoglobin plus emulsion after  $\text{NH}_4\text{OH}$  treatment; C, hemoglobin plus emulsion after  $\text{NH}_4\text{OH}$  and  $\text{H}_2\text{O}$  treatment. R, instrument reading.

Fig 2—Standard curve showing instrument readings (R) plotted against fat concentration in diluted emulsion. (Above R or 220 the curve is no longer linear.)

In an effort to make the method more sensitive without necessitating the use of larger blood samples, two changes were made. First the standard Klett tubes were replaced with microtubes, and second, filter No. 42 was used in place of No. 66. To remove any hemoglobin which might be present various oxidative reagents were tried and it was found that hydrogen peroxide was most satisfactory. The following experiment illustrates the effect of the peroxide treatment on the hemoglobin and the fat emulsion. Four milliliter aliquots of a diluted emulsion were placed in standard Klett tubes to which were added various quantities of a hemoglobin solution containing 29 mg of hemoglobin per milliliter. The tubes were brought to the 5 ml mark with water and the turbidities were read using filter No. 42. To each tube was added 0.05 ml of Superoxol and the tubes were heated in a water bath at 60° C for five minutes. After cooling to room temperature the turbidities again were read in the colorimeter. The results are shown in Fig. 1. It is necessary that the tubes be cooled to room temperature before turbidity readings are taken, otherwise the results will be significantly low.

*Adopted Procedure*—By means of a hemoglobin pipette 20 cmm of blood are collected and discharged into 2 ml of 5 per cent dextrose solution contained in a 13 by 100 mm Pyrex test tube and the pipette is rinsed in the usual manner. If any clotting occurs the sample should be discarded. The contents of the tube are well mixed by rotation and the tube is centrifuged at 1300 revolutions per minute for ten minutes. The supernatant is decanted into a Klett microtube, care being taken to avoid carry over of the sedimented cells and 0.05 ml of concentrated ammonium hydroxide is added, after mixing 0.05 ml of Superoxol is added. The tubes are heated in a water bath at 60 to 65° C for four minutes and then cooled to room temperature. Any moisture which has condensed on the sides of the tube is mixed in, and any bubbles of gas attached to the sides of the tube are removed by gentle tapping. The turbidity is read in the colorimeter using filter No. 42.

A standard curve is obtained by making various dilutions of the emulsion using 5 per cent dextrose solution as the diluent. Two milliliter quantities of each dilution are placed in the microtubes and the tubes are carried through the procedure outlined in the preceding paragraph. The turbidities obtained are plotted against the concentration of fat. Where greater accuracy is required the standard curve can be made with 15 ml samples, and in the procedure for the unknown, instead of simply decanting as much of the supernatant as possible a 15 ml aliquot can be taken. This would make the dilution effect of the reagents a constant throughout. In routine practice however it is sufficient to use the method outlined. A typical standard curve is given in Fig. 2.

*Comparison With Microoxidation Method*—A rabbit weighing 4310 grams was given an injection by ear vein of 12 ml of the emulsion in the course of two minutes. At intervals 2 ml samples of rural venous blood were taken and immediately heparinized. After centrifuging at 1300 revolutions per minute for fifteen minutes, 20 cmm of the plasma were added to 2 ml of 2 per cent dextrose solution contained in Klett microtubes. The tubes were carried

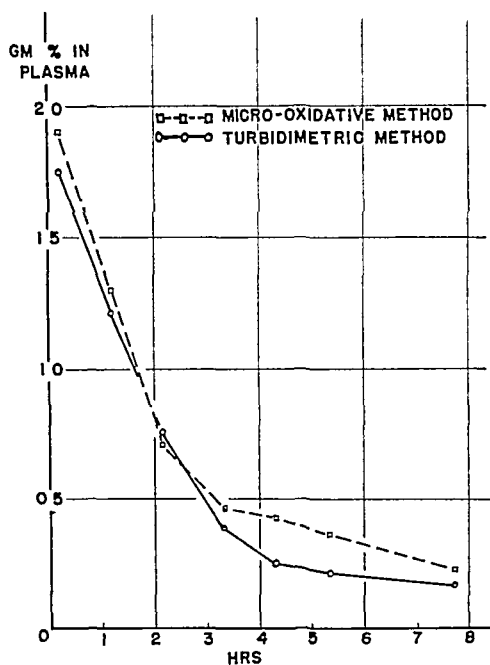


Fig 3

Fig 3—Comparison of microoxidation method and turbidimetric method of plasma fat following intravenous administration of fat emulsion

Fig 4—Fat tolerance curves of various species following intravenous administration of  $1\frac{1}{2}$  Gm of fat per kilo of body weight

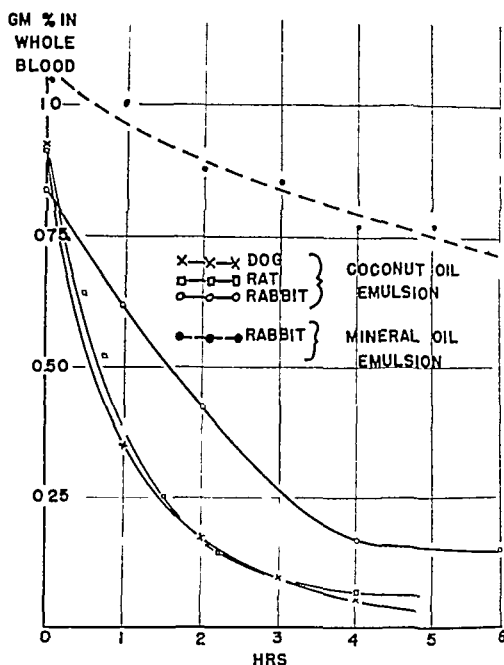


Fig 4

through the regular procedure and the turbidities were read in the colorimeter. Concurrently, suitable aliquots of each of the plasma samples were taken and carried through Boyd's modification<sup>3</sup> of Bloo's microoxidation method for total fatty acids plus cholesterol. The results of this experiment are given in Fig 3.

**Fat Tolerance Curves**—The tolerance curves of three species of animals to a 30 per cent coconut oil emulsion (Emulsion 35) and of one species (the rabbit) to a 30 per cent mineral oil emulsion were determined. Several rabbits, dogs, and rats each were given 5 ml of emulsion per kilogram of body weight at the rate of 2 ml per minute. The rabbits were injected by ear vein, the dogs by leg vein, and the rats by tail vein. Turbidity curves were obtained on venous blood using the procedure given previously. The results of these experiments are shown in Fig 4 where each curve represents one animal of each species.

#### DISCUSSION

The turbidimetric determination of infused fat in the blood following intravenous administration of a fat emulsion can be quickly accomplished with only 20 cmm of blood. It is essential that the emulsion be stable in the blood and to subsequent treatment with the reagents used. It is equally important that hemoglobin and extraneous turbidity be removed. As shown in Fig 1 and Table I these conditions have been adequately met. That the values agree well with a conventional microoxidation method is shown in Fig 2. It is to be ex-

pected that the curve obtained by turbidimetric means would be lower than that found by the microoxidation method, especially as the latter has been employed here, since total fatty acids plus total cholesterol have been determined. The turbidimetric method determines principally the infused neutral fat, because the normal turbidity of the plasma lipids is extremely low. That a change in instrument and conditions might allow the determination of the normal colloidal lipids in plasma is indicated by the report of Moreton.<sup>4</sup> In several instances chylomicriographs<sup>5</sup> have been determined simultaneously with the turbidity curves after a fat emulsion injection. Agreement between the resulting curves was good.

The reaction between the phosphatide and plasma which gives rise to the extraneous turbidity observed may be similar to that reported by Chargaff and Ziff<sup>6</sup> to occur between cephalin and basic proteins. A large percentage of the phosphatide is made up of cephalins, and *in vitro* tests have shown that when the pH of a mixture of plasma and phosphatide is lowered, a voluminous precipitate is formed. Neither the plasma nor phosphatide alone will form such a product. The resulting precipitate when washed with water, acetone, and petroleum ether was insoluble in water but readily dissolved in a weak ammonium hydroxide solution. Reprecipitation results if the solution is reacidified. While plasma protein is involved is unknown at present. In agreement with the findings of Chargaff and associates<sup>7</sup> is the fact that the phosphatide reacts with oxyhemoglobin and on acidification yields a reddish brown precipitate. The latter is soluble in dilute ammonium hydroxide and this may explain why, as shown in Fig. 1, the turbidity decreased slightly when  $\text{NH}_4\text{OH}$  was added even though no plasma was present.

TABLE I PER CENT RECOVERY OF FAT FROM MIXTURES OF PLASMA AND FAT EMULSION

TUBE	DILUTED EMULSION (ML.)	PLASMA* (ML.)	5 PER CENT DEATROSE (ML.)	TURBIDITY FADING†			
				BEFORE	AFTER $\text{NH}_4\text{OH}$ ADDITION	AFTER $\text{H}_2\text{O}$ ADDITION	RECOVERY (%)
1	0.2	--	9.8	190	190	187	--
2	0.2	--	9.8	188	188	184	--
3	0.2	0.2	9.6	196	190	187	101
4	0.2	0.5	9.3	176	205	195	101
5	0.2	0.8	9.0	186	214	197	--
6	0.2	1.0	8.8	194	216	202	98.1
7	--	0.5	9.5	18	18	8	--
8	--	1.0	9.0	37	37	20	--

\*Rabbit plasma.

†Instrument readings made with filter No. 4.

The removal of the hemoglobin by the hydrogen peroxide is complete when the amount of hemoglobin does not exceed that normally present. Where hemolysis is excessive recourse to filter No. 66 can always be taken. It is usually possible to avoid mechanical lysis of the cells. The fact that the peroxide treatment has no effect upon the emulsion is further indication of the latter's stability.

The term fat tolerance curve designates the rate at which infused fat leaves the blood stream and it is possible that like the glucose tolerance curve it may



become of diagnostic importance. Studies are now in progress on this phase of the problem. Such curves are a convenient guide in the use of fat emulsions for intravenous nutrition. It is of interest that the rabbit, which normally ingests little fat, cleared the infused fat at a slower rate than either the rat or dog whose diet is usually higher in fat (see Fig 4). The slow removal of the mineral oil emulsion is also of interest because the oil concerned is non hydrolyzable. Further work with this oil may reveal the reason for this slow removal. Unlike the blood lipid curve which results after oral fat intake, the curve obtained after a fat emulsion infusion is not subject to the influence of new lipid continually entering the blood. Perhaps by knowing the animals' ability to use infused fat, the curve obtained by oral fat ingestion could be corrected to give a clearer picture of the rate of absorption from the intestine. This would assume that normal blood fat and infused fat are handled by the body in the same manner.

#### SUMMARY

A simple turbidimetric method is described for determining the level of infused fat in the blood after intravenous administration of a fat emulsion.

The effects of hemoglobin and extraneous turbidity are discussed and the method of their removal is given.

The method is compared with the microoxidation method for blood lipids.

Fat tolerance curves are given for the dog, the rat, and the rabbit. The difference between the behavior of mineral oil and coconut oil in the rabbit is shown.

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## STUDIES ON THE CONGLUTINATION TEST IN ERYTHROBLASTOSIS FETALIS

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THE original method<sup>1</sup> (blocking test) of demonstrating univalent antibodies (blocking antibodies or glutinins) was indirect and therefore relatively insensitive. The conglutination test<sup>2</sup> for univalent antibodies, on the other hand, is a direct and more sensitive test and therefore has proved more satisfactory. While in saline media univalent Rh antibodies merely "coat" Rh positive cells by combining with the specific antigen without producing any visible reaction, in plasma or serum media clumping of the coated red blood cells occurs. As has been demonstrated in previous papers,<sup>3</sup> this clumping is not due to agglutination but to conglutination. The plasma contains a third component, conglutinin, a colloidal complex of plasma proteins, which is adsorbed by the specifically sensitized red blood cells, causing them to stick together. On the other hand, agglutination in saline media is as distinct as or more distinct than in plasma media because the agglutinins (bivalent antibodies) link the cells together directly without the intervention of any third component.

On the basis of these considerations, it was postulated that univalent antibodies are composed of smaller molecules than bivalent antibodies and therefore traverse the placenta more readily than the latter.<sup>4,5</sup> Ample direct and indirect evidence<sup>6,7</sup> confirming this prediction has now been obtained showing that univalent Rh antibodies as well as univalent alpha and beta and other antibodies pass through the placenta early in the third trimester of pregnancy and accumulate in the fetal body until the titers in the fetal and maternal plasmas become equal. Where the fetus is Rh positive or belongs to an incompatible A B group, the antibodies are first taken up by the red blood cells or tissues and only after these are coated do free antibodies accumulate in the plasma. In these cases if the baby is born alive, the red blood cells at birth are coated with univalent antibodies and it was found that this could be demonstrated simply by the conglutination technique.<sup>8</sup> One of the purposes of this paper is to describe the results obtained with this test in a series of cases of Rh sensitization, and to compare the test with the antiglobulin method of Coombs and coworkers<sup>9</sup> and Hill and Harberman.<sup>10</sup>

At this point it may be of interest to mention other predictions made on the basis of the conglutination theory,<sup>3</sup> which have been confirmed subsequently. A characteristic of erythroblastosis fetalis is that many babies appear normal or only mildly affected at birth but within a few hours jaundice becomes evident and rapidly increases in intensity, and the disease often terminates with the death of the infant within a day or two. Such infants usu-

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all present the postmortem findings of kernicterus and liver necrosis. This sequence of events occurs whether delivery takes place at term or whether the pregnancy is terminated prematurely by cesarean section or by induced labor. This indicates that the birth itself must precipitate the progress of the disease. It was reasoned that while the child was in utero the fetal cells did not clump because fetal plasma must be deficient or lacking in conglutinin. However, the profound physiologic changes occurring at birth could result in abrupt increase in the conglutinin content of the plasma causing the coated red blood cells to clump (by conglutination), thus blocking the circulation to vital organs. Direct measurements of the conglutinin content of fetal plasma confirmed this prediction that the amount present is small and that the conglutinin content increases after birth, though not to the level characteristic for adult plasma. It is interesting that complement has a similar development, considering that complement has an analogous role in serologic lysis to that of conglutinin in the conglutination reaction.

Still another surmise made in connection with the conglutination theory is that conglutinin is probably identical with or related to the  $\gamma$  protein of Pedersen.<sup>11</sup> According to Pedersen  $\gamma$  protein is a reversibly dissociable complex of albumin and globulin, present only in concentrated plasma or serum because slight dilution with aqueous solutions causes it to dissociate into its constituent molecules of albumin and globulin. In parallel with the behavior of  $\gamma$  protein, we found that relatively slight dilution of plasma with isotonic aqueous solutions causes it to lose its conglutinating activity. Conversely, we reasoned that it should be possible to produce conglutinin by mixing solutions of albumin and globulin. We did find that when a 4.6 per cent solution of human globulin and a 12.5 per cent solution of human albumin, both of which had little or no conglutinating activity, were mixed so as to produce a solution with a total protein content and albumin:globulin ratio equivalent to that of normal blood serum, the resulting mixtures proved to have a high conglutinating activity, even exceeding that of normal plasma. Here again the analogy between conglutinin and complement holds, since this may be compared with the experiments with so called midpiece and endpiece.

Obviously, the sensitivity of the conglutination test will depend not only on the titer of the univalent antibody but also on the quality of the conglutinin. While the original description of the test called for the use of inactivated serum, we soon adopted the use of oxalated plasma<sup>4</sup> because the latter has a higher conglutinating activity and is relatively free of rouleau forming properties. Moreover, it was found that while pure albumin itself was inferior to oxalated plasma, nevertheless the addition of small amounts of albumin to plasma enhanced the latter's conglutinating activity,<sup>5</sup> presumably because the added albumin combined with the natural conglutinin in the plasma to form a more active complex. Based on this observation, the albumin plasma conglutination test was devised,<sup>3</sup> which must not be confused with the albumin test of Drimond and Denton<sup>12</sup> since the latter calls for a pure 25 per cent solution of human albumin or a pure 30 per cent solution of bovine albumin without any plasma.

#### MATERIALS AND METHOD

The material which formed the basis for the present study consisted mainly of infants born to Rh negative women who had been studied during the prenatal period for the presence of Rh sensitization. Our series is a selected one in that many of the pregnant women were referred to us either because they had previously had erythroblastotic infants or because they had been found to be Rh negative in the course of routine Rh antibody examinations. In a few cases we were not called to see the affected infants until after birth, either because antenatal tests had not been done or because the mother had been incorrectly typed during pregnancy as Rh positive.

At the first interview, the expectant mother's blood was completely classified for blood group, MN type, and Rh Hr type, and the same tests were made on the husband to determine, if possible, whether he was homozygous or heterozygous for the Rh factor. Moreover, antibody tests were done periodically on the prospective mother's serum, and, based on the Rh Hr tests and antibody titrations, predictions were ventured as to the type and severity of

the disease in the infant. The affected infants were mostly treated by exchange transfusion, as will be reported elsewhere.<sup>13</sup> As also will be shown in a separate report the manifestations closely corresponded to the predictions and in a number of cases the infants were delivered prematurely by cesarean section or by induced labor in order to prevent stillbirths and to treat the infants by exchange transfusion.<sup>14</sup> Where possible samples of the cord blood were obtained in order to test the red blood cells for coating with univalent antibodies and to examine the infant's serum for free Rh antibodies and to determine the icterus index. In the few cases where the cord serum could not be obtained because the infant was seen after birth, these determinations were carried out on the first sample obtained at the exchange transfusion.

The Rh antibody titrations on the maternal and infant sera were carried out by the agglutination and albumin plasma conglutination methods, as described in previous papers.<sup>3, 4</sup> In some cases the sera were also titrated by the plasma conglutination method and blocking method but the titers are not included in Table I in order to avoid complicating the presentation. All titrations were done at least two times against test cells of types Rh<sub>1</sub> and Rh and the titer values obtained were averaged. In testing for coating of the fetal cells 2 drops of a 2 per cent saline suspension of the red blood cells were centrifuged and the supernatant fluid removed completely and replaced by a drop of compatible plasma or albumin plasma mixture.<sup>15</sup> The red cells were resuspended and after incubating for forty five minutes at body temperature the preparation was examined for the presence of clumping. The saline suspension itself of the infant's cells invariably showed no trace of clumping. Control tests were always carried out on normal red cell suspensions to show that the plasma albumin mixture was incapable of clumping such cells. For the antiglobulin test an anti human precipitin serum was absorbed with washed packed, and pooled A and B cells to remove all heteroagglutinins. The actual tests were carried out by adding this reagent (diluted 1:2 or higher depending on its titer) to 1 drop of thrice washed saline suspension of infant's red blood cells, readings being taken after forty five minutes of incubation at body temperature.

The icterus index determinations were made by the acetone precipitation method.

Titration of sera containing univalent antibodies by the antiglobulin technique were carried out in the following manner. To a series of tubes containing 1 drop each of a series of progressively doubled dilutions of the serum 1 drop of a 2 per cent saline suspension of Rh positive cells was added and the mixtures allowed to react in the water bath for one hour. The red cells were then washed three times with saline solution. After the third washing the sediments were resuspended in 1 drop of saline solution, and 1 drop of the absorbed anti human precipitin serum was added. The mixtures were incubated for another hour after which the reactions were read.

## RESULTS

Nine different sera from sensitized Rh negative women were titrated by the blocking, plasma conglutination, albumin plasma conglutination, and anti globulin techniques in order to compare the relative sensitivities of these methods of detecting univalent Rh antibodies (see Table I). Two of the sera tested (Sera 8 and 9) contained agglutinins as well as univalent antibodies. In accordance with our previous report,<sup>3</sup> the blocking test gave the least sensitive results, and the plasma conglutination test was on the average about ten to fifteen times as sensitive as the blocking test, while the albumin plasma conglutination test was on the average about four times as sensitive as the plasma conglutination test. As shown in Table I, the antiglobulin technique gave results roughly corresponding to those obtained by the plasma conglutination test, being less sensitive than the albumin plasma method.\* It is felt that the

\*The experiments were repeated several times using at least three different precipitin sera for the antiglobulin test always with similar results.

TABLE I COMPARISON OF THE RELATIVE SENSITIVITIES OF THE CONGLUTINATION AND ANTIGLOBULIN METHODS OF TITRATING UNIVALENT ANTIBODY

SERUM	ANTIBODY TITERS (UNITS*) BY THE METHODS OF				
	AGGLUTINATION	BLOCKING	PLASMA CONGLUTINATION	ALBUMIN PLASMA CONGLUTINATION	ANTIGLOBULIN TECHNIQUE
1	0	15		520	20
2	0	3	23	135	47
3	0	1½	2½	45	6
4	0	0	4	44	6
5	0	0	5	36	4
6	0	1	25	28	5
7	0	½	11	17	12
8	7	0	20	62	7
9	12	0	12	28	5

\*The figures given represent the average of two or more titrations

albumin-plasma method is to be preferred to the antiglobulin technique, since the former is much simpler to perform and gives more sensitive results

In Table II are summarized a series of cases in which Rh-negative mothers and their infants were studied serologically for evidence of Rh sensitization. For clarity in discussing the findings, the cases have been divided into four groups as follows:

1 Eleven cases in which the maternal serum contained univalent antibodies without detectable agglutinins. They are arranged according to the titer of the antibodies immediately prior to or following delivery.

2 Eight cases in which the maternal serum contained Rh agglutinins with or without Rh univalent antibodies.

3 Three cases of sensitized Rh-negative women who gave birth to normal Rh-negative infants.

4 Four control cases of nonsensitized Rh-negative women with normal Rh-positive infants.

We have omitted from this table those cases in which the mother was sensitized to the A and B factors as well as to the Rh factor, these complicated cases of double sensitization will be discussed in a later paper. Two cases (Cases 16 and 25) have been included in the table in which the baby's blood group was incompatible with the mother's, but in these cases the maternal anti-A and anti-B titers (by the conglutination as well as by the agglutination technique) were lower than average, showing that the mothers were not sensitized to the A or B factors.

It would be expected that in all cases where the maternal serum contains a high titer of univalent antibody the infant would be born with its red cells completely coated with blocking antibodies. Where the maternal antibody titer is low, on the other hand, the red cells of the erythroblastotic infant would be expected to be only partly coated. As shown in Table II, this prediction was actually fulfilled in our series of cases. Thus, the Rh-positive red cells of the erythroblastotic infants of the most strongly sensitized mothers (Cases 1, 2, 3, 4, 5, 6, 7, 10, 12) failed to clump in anti-Rh<sub>0</sub> agglutinating serum, due to complete blocking of the cells. Moreover, with two exceptions (Cases 1 and 3) these

TABLE II RESULTS OF SERIOLOGIC TESTS ON A SERIES OF RH NEGATIVE WOMEN AND THEIR INFANTS

CASE	MOTHER'S BLOOD TYPE	Rh ANTIBODIES IN MATERNAL SERUM (UNITS)		BABY'S BLOOD TYPE	COATING TEST ON INFANT'S RED CELLS			TITRATION OF FREE Rh ANTIBODIES IN INFANT'S SERUM (UNITS)			ICTERUS INDEX (CORD SERUM)
		AGGLUTINA TION METHOD	ALBUMIN CONGLU TIN ATION METHOD		IN PLASMA	IN ALBUMIN PLASMA	COOMBS TEST <sup>1</sup>	AGGLUTINA TION METHOD	ALBUMIN PLASMA CONGLU TIN ATION METHOD		
1*	A MNrh	0	1400	AMRh <sup>+</sup> †	-	-	++	0	400		30
2	A Mrh	0	112	AMRh †	++	++	++	0	4		64
3*	A Nrh	0	45	OMNRh rh†	++	-	++	0	18		44
4	BMNRh	0	40	OMNRh rh†	++	++	++	0	1½		50
5†	A MNrh	0	40	OMRh †	++	++	++	0	2		70†
6†	A Mrh	0	39	OMNRh <sub>2</sub> rh†	++	++	++	0	3		64+
7	A Nrh	0	32	AMNRh rh†	++	++	++	0	1½		20
8	A Mrh	0	30	AMRh rh	++	++	++	0	4		20
9	OMNRh	0	2j	OMNRh rh	++	++	++	0	0		60
10	AMNRh	0	16§	AMRh rh†	++	++	++	0	0		3j
11	AMNRh	0	1	ONRh rh	-	++	++	0	0		14
12	OMNRh	42	40	OMNRh rh†	++	++	++	0	3		-0
13†	OMNRh	20	70	OMNRh rh	++	++	++	0	0		60†
14†	OMrh	25	40§	OMNRh rh	-	-	++	0	0		45†
15	OMrh	22	20	OMNRh <sub>2</sub>	-	-	++	0	1½		12
16	BMrh	4	12	ABMNrh <sub>2</sub> rh	+	Trace	±	0	0		12
17	A <sub>2</sub> Mrh	7	10	AMRh rh	-	++	-	0	0		-4
18	A BMNRh	1	2	BMRh rh	-	++	-	0	0		24
19†	BMNRh	1	1	ONRh rh	-	++	±	0	0		112†
20	ANrh	0	22	ANrh	-	-	-	0	20		10
21	AMrh	0	7	OMrh	-	-	-	0	7		10
22	AMrh	24	11	AMNRh	-	-	-	0	2½		10
23	OMrh	0	0	OMRh rh	-	-	-	0	0		6
24	BMNRh	0	0	BMNRh rh	-	-	-	0	0		8
25	OMrh	0	0	AMRh rh	-	-	-	0	0		10
26	AMrh	0	0	AMNRh rh	-	-	-	0	0		14

\*These patients died despite treatment by exchange transfusion

 †These blood samples failed to clump in anti Rh<sub>0</sub> agglutinating serum due to blocking

‡These patients were seen for the first time twenty four hours after birth

§These titers represent results of plasma conglutination tests since the cases were seen before the albumin plasma technique was developed

||These babies were treated by exchange transfusion and recovered

cells clumped (conglutinated) when suspended in compatible plasma or in albumin-plasma mixture

The failure of the red cells of the infants of Cases 1 and 3 to conglutinate is contrary to expectation and calls for an explanation. The following plausible hypothesis suggests itself. As has already been demonstrated,<sup>2, 5</sup> fetal plasma is deficient in conglutinin in comparison with adult plasma. Also, there is reason to believe that fetal conglutinin differs in quality as well as quantity from adult conglutinin. Thus one may postulate that fetal plasma contains conglutinoid, a substance analogous to so-called complementoid.<sup>16</sup> In cases in which the fetal red cells are strongly sensitized by univalent antibodies, they will absorb conglutinoid from the fetal plasma, but conglutinoid, unlike conglutinin, fails to clump the cells, just as complementoid is adsorbed by sensitized cells but fails to lyse them. When an infant's sensitized red cells, which have adsorbed conglutinoid, are suspended in plasma they will fail to clump or clump only feebly because the conglutinoid will block the adsorption of conglutinin. Supporting this concept is the observation that such red cells are clumped strongly by antiglobulin serum even though the antiglobulin technique is ordinarily less sensitive than the albumin-plasma conglutination technique for demonstrating univalent antibodies, as has already been pointed out (Table I).

In accordance with expectations, when the maternal univalent antibody titer is very high, not only are the infant's red cells completely blocked but also free circulating antibodies can be demonstrated in the fetal plasma. Where the maternal antibody titer is low, the infant's red cells are not blocked, but partial coating of the red cells can be demonstrated by the more sensitive conglutination technique (see Cases 8 and 9, Table II).

Where the maternal serum contains bivalent Rh antibodies, one would expect that the infant's red cells at birth would not be coated with univalent Rh antibodies. As is shown in Table II, this was true in only half the cases in our series. To account for coating of the infants' cells in Cases 12, 13, 16 and 18, one must postulate that the maternal serum contained univalent as well as bivalent Rh antibodies but that the former were weaker so that they were masked by the agglutinins. In support of this idea, it may be pointed out that in two cases (Cases 13 and 16) the infants' sera contained free univalent Rh antibodies.

Of special interest are Cases 20, 21, and 22 where sensitized Rh-negative women gave birth to Rh-negative children. As expected, none of these infants were erythroblastotic. In two of the cases where the maternal serum contained pure univalent antibodies, the infants' sera contained univalent antibodies of equal titer. In the third case, where the maternal serum contained moderately strong (24 units) Rh agglutinins, the infant's serum contained no Rh agglutinins but univalent Rh antibodies of  $2\frac{1}{2}$  units. In the latter case, presumably, the maternal serum also contained Rh univalent antibodies of  $2\frac{1}{2}$  units titer, but these were masked by the Rh agglutinins. Thus, the placenta acts as a semipermeable membrane which permits the free passage of univalent antibodies but holds back bivalent antibodies.

In the four control cases\* in which the maternal serum contained no Rh antibodies, the infants, though Rh positive were not erythroblastic and the red cells showed no evidence of coating

Of considerable importance is the correlation between Rh antibody titer and the severity of the disease in the fetus and infant. Where the maternal serum contains univalent antibodies which readily pass the placental barrier a good correlation is to be expected. On the other hand if the maternal serum contains only bivalent antibodies, the titer may be less important than other factors such as an accidental defect in the placental barrier or increased intrauterine pressure causing the antibodies to enter the fetal circulation.<sup>6</sup> These expectations have been fulfilled in our series of cases. When the maternal serum contains potent blocking antibodies the Rh positive fetus is invariably delivered as a macerated stillbirth such cases were not included in Table II because the fetal blood in these cases was not suitable for examination. It is significant that of the cases listed in Table II the four babies that died in spite of treatment came from the mothers with the highest titers of univalent antibodies. Similar observations showing the correlation between quality and quantity of antibody and prognosis have been made by Sacks and associates<sup>1</sup> and by Torregrossi<sup>18</sup> in conformity with Wiener's theory,<sup>6</sup> and more recently by Davidson.<sup>19</sup>

#### SUMMARY

Of the various methods of demonstrating univalent Rh antibodies, the blocking test is the least sensitive the plasma agglutination test is about ten times as sensitive as the blocking test, and the albumin plasma agglutination test is about four times as sensitive as the plasma agglutination method. The antiglobulin technique is about equal in sensitivity to the plasma agglutination technique.

When the maternal serum contains univalent Rh antibodies, these antibodies pass through the placenta and coat the infant's Rh positive red cells as can be demonstrated by the blocking agglutination, and antiglobulin techniques. The higher the titer of the univalent antibody in the maternal serum, the more completely are the fetal red cells coated and the more likely is the fetal plasma to contain free Rh antibody.

When the maternal serum contains bivalent antibodies the fetal red cells will not be coated unless the maternal serum also contains univalent Rh antibodies.

When an Rh negative woman with univalent Rh antibodies has an Rh negative baby the baby will not be erythroblastic but its plasma will contain univalent Rh antibodies equal in titer to that of the maternal plasma at the time of birth.

Rh agglutinins usually are not demonstrable in the plasma of an Rh negative infant even when the maternal serum contains a high titer of these antibodies.

\*Many more such cases have been studied but four are sufficient for purposes of illustration.



The placenta acts as a semipermeable membrane which permits the passage of univalent antibodies but holds back bivalent antibodies

Infants of nonsensitized Rh-negative women are not erythroblastotic provided other sensitizations are also excluded, for example A-B sensitization

Where the maternal serum contains univalent Rh antibodies, the severity of the manifestations in the erythroblastotic infant is roughly proportional to the antibody titer

Evidence is presented suggesting the presence of a substance in fetal plasma, conglutinoid, which is adsorbed by cells sensitized by univalent antibodies but fails to clump them. Conglutinoid seems to be capable of blocking the action of conglutinin in much the same way that so-called complementoid blocks the action of complement

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## A STUDY OF CHOLINESTERASE ACTIVITY OF THE BLOOD OF PATIENTS WITH PERNICIOUS ANEMIA

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IN 1940, Sabine<sup>1</sup> reported red cell esterase rises with concomitant plasma and whole blood esterase reduction in two phlebotomized dogs. In a study<sup>1</sup> of fifteen patients with various types of anemias she reported low plasma esterase activity in debilitated and comatose states. The red cell esterase activity in these patients did not parallel that of the plasma. In general, the red cell and plasma activities were lower in those patients with hematocrits less than 30 per cent and there was normal activity where the hematocrit was above this value. Three patients with pernicious anemia in relapse receiving liver therapy were studied during their recovery. Initially, the whole blood, red cell, and plasma esterase activities were low. During treatment the cell esterase returned to normal levels in four to nine days. This was followed by a further increase in activity and a decline to normal levels in about six weeks. The plasma esterase responded much more slowly, rising to normal in about six weeks.

Previously, Antopol<sup>2</sup> and associates reported a general lowering of serum esterase in patients with secondary anemias and acute hemolytic anemia. Hall and Lucas<sup>3</sup> found no correlation between the serum esterase activity and the hemoglobin concentration, red cell and white cell counts. Ginsberg, Kohn, and Neeheles<sup>4</sup> found that washed pus cells had no esterase activity. Milhorat<sup>5</sup> reported lowered serum esterase in patients with leucemia. He noted no correlation between the serum esterase and the hemoglobin or between the serum esterase and the red cell count of the peripheral blood. There is general agreement that the plasma or serum cholinesterase activity is decreased in debilitated states<sup>1, 5, 7</sup> and in liver and biliary tract diseases.<sup>2, 6, 7</sup> Low normal values are found in association with acute infections.<sup>3</sup> Hall and Lucas<sup>3</sup> could not demonstrate any relationship between the serum esterase activity and age, sex, diet, body activity, heart rate, or blood pressure in normal and pathologic sera. The role of cholinesterase in myasthenia gravis and the neuromyopathies is not clear. In these diseases the red blood cell esterase is reported to be normal while the plasma esterase is generally reduced.<sup>7, 8</sup>

Since 1944 a series of articles has been published by Davis and co-workers<sup>10, 14</sup> concerning the experimental production of a hyperchromic anemia in dogs which responds to antipernicious anemia therapy. Interestingly enough one report<sup>13</sup> contains a description of nervous system changes similar to the neuropathologic changes noted in human pernicious anemia. Concomitant

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with the production of the anemias, the cholinesterase activity of the dog sera fell to a low value. With the institution of antipermeious anemia therapy, the red cell count, hemoglobin and esterase activity of the serum returned to normal levels. In 1946, Davis<sup>11, 12, 14</sup> reported that folic acid could be used in place of liver extract. He also stated that folic acid administered to normal human subjects increased the serum esterase activity significantly within five hours. During the same year, he observed an increase in an acetylcholine-like substance in the blood of patients with pernicious anemia which returned to normal levels after antipermeious anemia therapy.<sup>12</sup> At this time he noted that the serum esterase activity paralleled the rising red cell count.

This report is concerned with the study of the cholinesterase activity of whole blood, red cells, and plasma in patients with pernicious anemia treated with folic acid and with combined folic acid and liver extract therapy.

#### METHODS

Seven patients with pernicious anemia in relapse were studied. The diagnosis was made on the basis of the peripheral blood picture, a megaloblastic bone marrow, the absence of free hydrochloric acid in the stomach, the absence of gastrointestinal disease after fluoroscopic and x-ray study, and guaiac negative stools. A reticulocytosis following adequate antipermeious anemia therapy was always noted.

Base lines for reticulocytes, hemoglobin, red cells, and cholinesterase activity were established before treatment was given. Cholinesterase activity was determined in the oxalated venous blood before and during the course of therapy.

Cholinesterase activity was measured by the method described in a previous report.<sup>15</sup>

#### CASE REPORTS

**CASE 1**—M. F., a 70 year old white woman, known to have pernicious anemia of several years' duration, sought treatment because of weakness, dyspnea, and sore tongue. Because of liver sensitivity she had had no therapy for the previous four months. Prior to that, treatment had been intermittent and irregular. Physical examination revealed a sore red tongue. The liver and spleen were not palpable. The gait was normal and the Romberg was negative. The knee jerk reflex was hyperactive and the Babinski sign was bilaterally positive. Position and vibratory sense were normal.

**Laboratory Data**—The stools were negative for occult blood. Gastric analysis revealed achlorhydria after histamine. The cephalin cholesterol flocculation was 1 plus and the icterus index was 10. The blood urea nitrogen was 15 mg and the blood sugar was 90 mg, per 100 ml of blood. The urine was normal. The bone marrow was not studied. Peripheral blood counts are shown in Table I.

The patient was given 20 mg of folic acid by mouth daily in the form of an elixir. Seventy-two hours later improvement of appetite and general status was noted. In one week the tongue symptoms were relieved. After three weeks, neurological examination disclosed no abnormalities.

Before treatment the cholinesterase activity of the blood was low (about one half the normal range). On the fourth day of therapy there was a simultaneous rise in the reticulocytes and an increase of the mean cell volume and mean cell esterase. Reticulocytosis was maintained for sixteen days. The corpuscular esterase activity reached a peak of 120 ( $\times 10^{-10}$ ) units (normal, 619 to 869 [ $\times 10^{-10}$ ] units) on the twentieth day of treatment and returned to normal levels about the thirty-sixth day. The mean corpuscular volume tended to parallel the reticulocyte curve, reaching a peak on the eighth day and returning to normal levels on the thirty-sixth day. The plasma esterase values remained constant throughout.

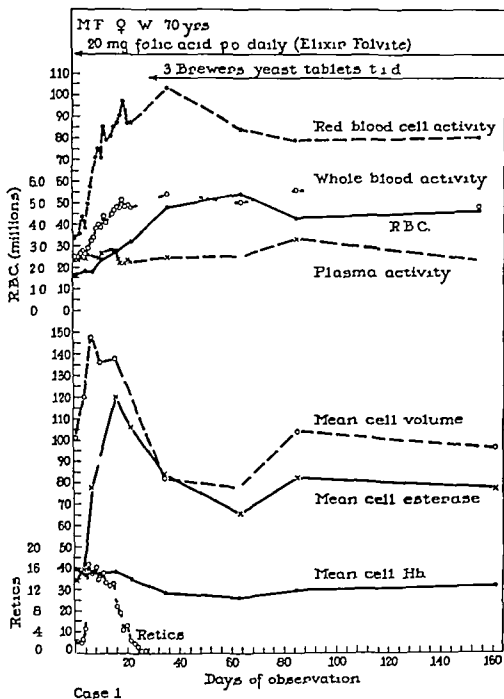


Fig 1

The erythrocyte count was not significantly increased until the eleventh day. Mean corpuscular hemoglobin gradually decreased reaching the normal range about the thirty-sixth day.

CASE 2—M P, a 73-year-old Negro woman was admitted to Kings County Hospital complaining of weakness and weight loss of three months duration. There was no history of bleeding. Physical examination revealed an extremely pale malnourished Negro woman. She was disoriented, incoherent and belligerent. The only other positive finding was bilaterally hyperactive knee jerk reflexes.

**Laboratory Data**—The stools were negative for blood and gastric analysis revealed histamine refractory achlorhydria. A gastrointestinal series and a barium enema study disclosed no abnormalities. The total blood proteins were 5.8 Gm. per 100 milliliters. The other blood findings per 100 ml were: calcium, 9.8 mg; phosphorus, 3.5 mg; urea nitrogen, 15 mg; blood sugar, 86 milligrams. The alkaline phosphatase was 4.1 units and the icterus index, 1.9. The bone marrow showed 30 per cent megaloblasts. The peripheral blood picture is shown in Table I.

Treatment of the patient was begun and 1 unit of liver extract combined with 0.3 mg of folic acid was given intramuscularly daily. For the first twenty-two hours sedation was required. A diarrhea was noted which persisted for the following ten days. After ninety-six hours the appetite was improved and general clinical improvement was noted. The reticulo-

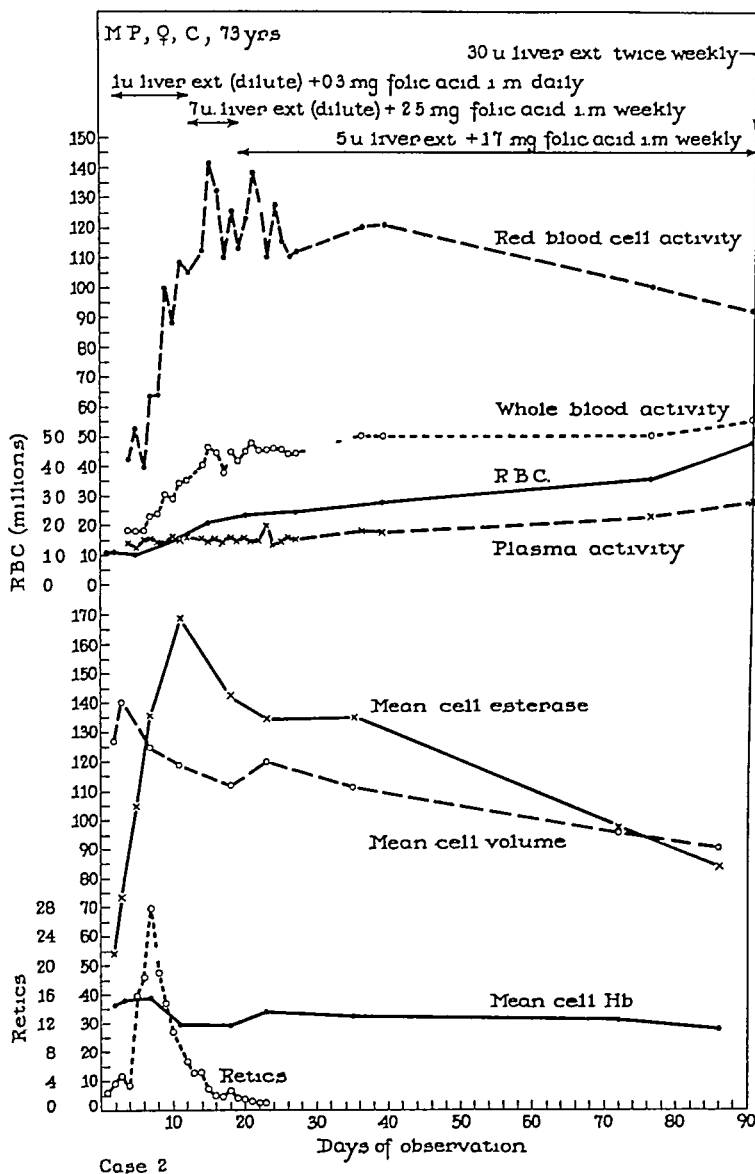


Fig 2

cyte response and the cholinesterase activity are shown in Fig 2. The patient made an uneventful recovery and on discharge was instructed to take 30 units of liver extract twice weekly.

The initial esterase values were obtained on the second day of therapy and were low (about one half the average normal value). On the fifth day significant increases in red cell esterase and in the number of reticulocytes were noted. The erythrocyte count rose and the mean corpuscular volume and hemoglobin started to fall. The mean cell esterase activity continued to rise, reaching a peak on the eleventh day. The reticulocyte curve returned to normal about the eighteenth day. The corpuscular esterase fell more slowly, it was abnormally high after 36 days, but returned to normal by the seventieth day. The plasma esterase remained constant for twenty-five days, then rose slowly, paralleling the erythrocyte increase.

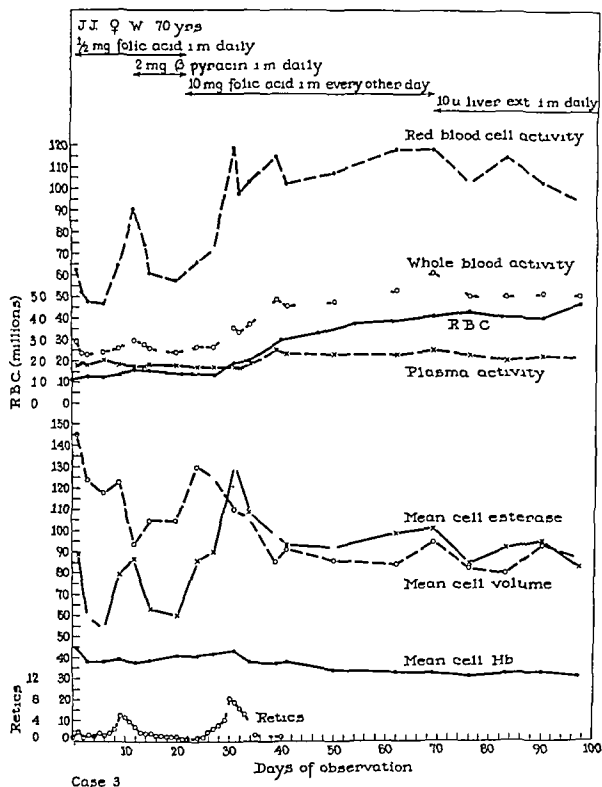


Fig 3

CASE 3\*—J J, a 70 year old white woman, was admitted to the Kings County Hospital complaining of progressive dizziness and tinnitus of four months duration. One week before admission weakness, blood tinged diarrhea, dysuria and polyuria were noted. A history of inadequate diet and the daily consumption of a pint of liquor for the past thirty years was obtained. On physical examination the only positive findings were a blowing apical systolic murmur, moderate right costovertebral angle tenderness, and external hemorrhoids.

**Laboratory Data**—A routine blood count revealed macrocytic hyperchromic anemia and 2 per cent megaloblasts in the peripheral blood. Bone marrow aspiration was confirmatory and a megaloblastosis of 32.5 per cent was noted. Gastric analysis was done and a histamine refractory achlorhydria was found. The chest x-ray and gastrointestinal series disclosed no abnormalities. Urine examination showed many white blood cells with clumping. The blood chemistry was normal.

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The patient was disorderly on admission and on the next day was disoriented and psychotic. She was given 0.5 mg of folic acid intramuscularly daily for twelve days. At this time 2 mg of beta pyracin were added to the daily dose. There was no clinical response, but a reticulocytosis of 5.2 per cent was observed. After ten days the daily administration of 10 mg of folic acid intramuscularly was started and a clinical and hematologic response followed. Nineteen days later, because of continued disordered sensorium, therapy was changed to the daily administration of 10 units of liver extract by the intramuscular route. After eighteen days of liver therapy the sensorium cleared and the patient made an uneventful recovery.

The initial esterase values were low, averaging 5 units of red cell esterase, 6 ( $\times 10^{-10}$ ) units of corpuscular esterase, and 1.8 units of plasma esterase. Following inadequate therapy with folic acid and beta pyracin, red cell and corpuscular esterase activities rose to a peak of about 9 units (in normal range) on the twelfth day and then fell rapidly to 6 units on the twenty-third day. A reticulocyte peak of 5.2 per cent was noted on the ninth day. The reticulocytes dropped to 1.8 per cent on the eleventh day and remained at about 1 per cent until seven days after folic acid in 10 mg doses was given, when another peak of 8.2 per cent was noted. The number of erythrocytes rose slightly during the first period and then fell off to pretreatment levels until effective folic acid therapy was given. Nine days later the red cell and corpuscular esterase rose sharply to a peak of 11.7 units and 13.0 ( $\times 10^{-10}$ ) units, respectively. The plasma esterase remained relatively constant throughout.

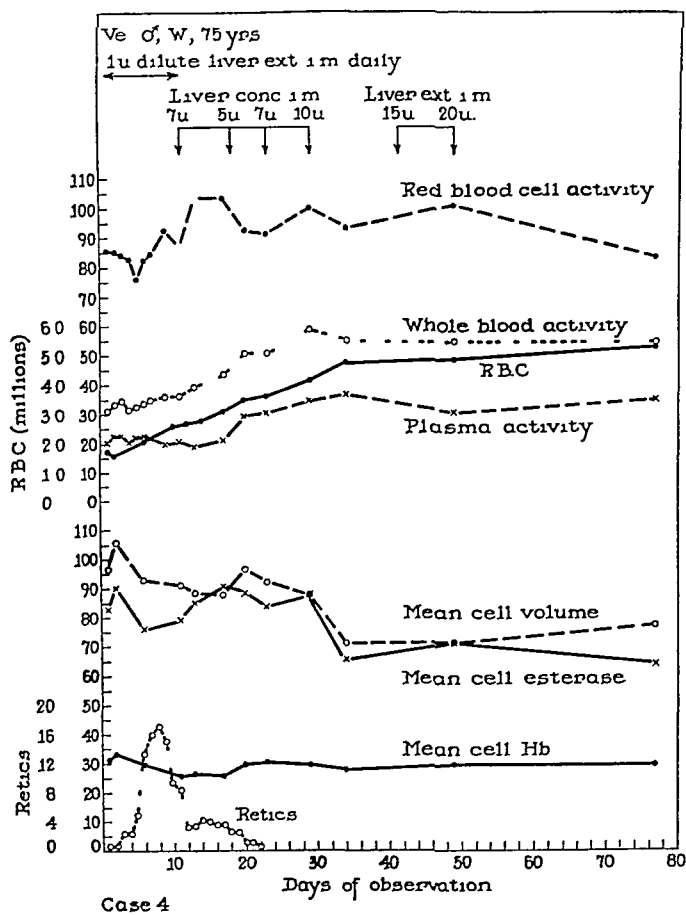
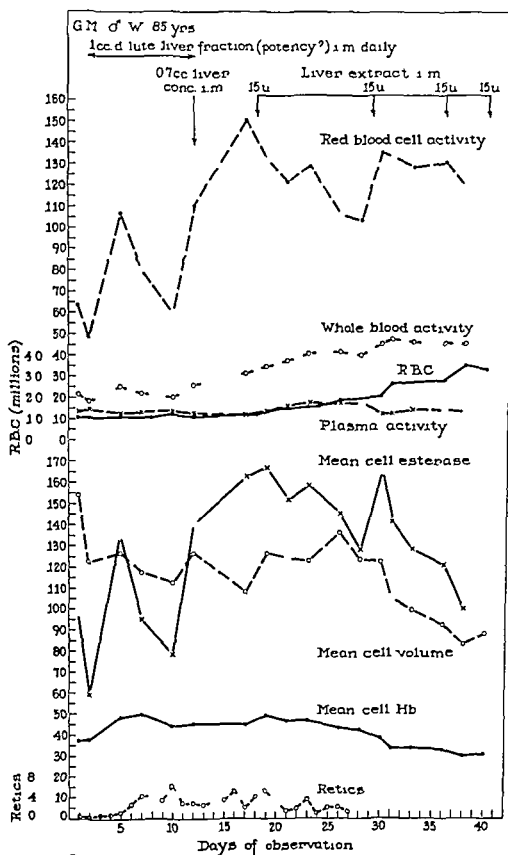


Fig 4

CASE 4—Ve, a 75 year old white man who was seen by one of us (L. M. M.), complained of weakness and loss of appetite during a period of one year. Six months previously he had been given a transfusion for "anemia" but no other therapy. Physical and neurological

examinations disclosed no abnormalities. A gastrointestinal series was normal and there was no blood in the stools. The blood findings were nonprotein nitrogen, 27 mg, and the fasting sugar, 90 mg per 100 ml, icterus index 8. The patient was treated with 1 unit of liver extract daily for ten days and then single injections of liver extract in equivalent unit dosage were given at approximately weekly intervals.

Initially the red blood cell and mean corpuscular esterase activities were within normal limits. The plasma activity was only slightly decreased. Following liver therapy there was a small increase in the erythrocyte esterase activity. A maximal value was attained on the sixteenth day. This value was sustained for an equal period of time and then fell to a lower plateau within the normal range. The plasma esterase rose to normal on the nineteenth



Case 5

Fig 5



day On the fourth day after therapy, a reticulocytosis was noted which reached a peak of 17.2 per cent on the seventh day The erythrocyte count rose steadily as is shown in Table II

CASE 5—G M, an 85 year old white man, was admitted to the Psychiatric Pavilion of Kings County Hospital because he was paranoid, abusive, hyperemotional, and unpredictable in his actions He was transferred to the Medical Service when a severe anemia was found Physical examination disclosed a well nourished man with a lemon yellow tint to the skin, icteric sclerae, and pale conjunctivae Neuropsychiatric examination elicited gross memory defect, lack of insight, and disorientation as to time and place

*Laboratory Data*—The urine analysis disclosed no abnormalities Histamine refractory achlorhydria was found The stools were free of blood The cephalin flocculation was negative and blood cholesterol was 153 mg per 100 milliliters A bone marrow aspiration revealed sheets of megaloblasts The peripheral blood findings are shown in Table I

The patient was difficult to manage and required restraint He was treated with 1 unit of liver extract intramuscularly daily, and a mild clinical but no hematologic response was noted Ten days later he was given 10 units of liver extract After six days he received fifteen units of liver daily intramuscularly There was clinical improvement, but a submaximal reticulocytosis was obtained The patient's mental status remained impaired and on the fortieth day of observation the patient was transferred to the psychiatric ward

The initial mean corpuscular esterase and red cell esterase activities were in the normal and low normal range, but the plasma activity was about one half of normal values Three days after the beginning of liver extract therapy the reticulocyte count rose, reaching a plateau of 4 to 6 per cent which was sustained for twenty two days The red cell esterase increased rapidly to 13.5 ( $\times 10^{-10}$ ) units on the third day and then fell abruptly to the initial levels on the eighth day There was a secondary rise to 14 ( $\times 10^{-10}$ ) units two days later, with a peak of 16.5 ( $\times 10^{-10}$ ) units on the sixteenth day The red cell esterase values then slowly fell toward lower but normal levels The plasma activity was constant and low throughout the period of observation The mean cell volume was above normal for thirty days and then returned to normal The red cell count remained stationary at about 1,000,000 per c mm for sixteen days and then rose slowly toward normal This erythrocyte response was observed six days after the second red cell esterase rise

CASE 6—A P, a 50 year old white woman known to have pernicious anemia, was admitted to Kings County Hospital for weakness and pallor of six months' duration She had been treated fifteen months previously for pernicious anemia in relapse and had responded well to 0.5 units of liver and 10 mg of folic acid intramuscularly daily She stopped all medication eight months before the present admission Physical examination was negative

*Laboratory Data*—A gastrointestinal series was negative and the stools were negative for blood The cephalin flocculation was 1 plus, the alkaline phosphatase, 4 units, and the icterus index, 6 The blood total protein was 6.2 Gm per 100 milliliters The urinary urobilinogen was positive to a dilution of 1:10

The patient was treated with an experimental liver fraction without hematologic or clinical response After five days, oral treatment with 1 unit of liver and 5 mg of folic acid was started Within forty eight hours there was marked clinical improvement The patient went on to make an uneventful recovery The peripheral blood picture and the cholinesterase response are shown in Table II

The initial red cell esterase values were only slightly below the normal range and rose rapidly to normal following the injection of an experimental liver fraction There was no reticulocytosis and no erythrocyte rise was noted during this period Three days after adequate therapy with liver and folic acid was instituted a reticulocytosis ensued A peak of 24 per cent occurred on the seventh day On the thirteenth day, following combined oral liver and folic acid the mean cell cholinesterase activity rose above normal and reached a peak of 10.8 ( $\times 10^{-10}$ ) units on the sixteenth day The red cell esterase gradually fell to normal values by the forty second day The erythrocyte count remained unchanged until eleven days

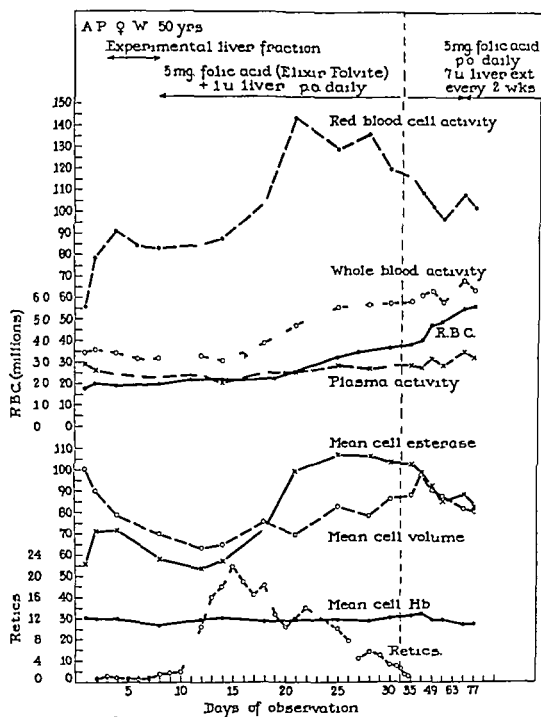


Fig 6

after combined therapy was given and then slowly rose to normal levels. There was an equivocal rise of the plasma activity seventeen days after the institution of combined therapy.

**CASE 7**—M O, a 56 year old white man, was admitted to Metropolitan Hospital for anemia of one year's duration, anorexia, weakness, dyspnea of effort, and dysphagia of four months' duration. Diarrhea for three weeks prior to admission was also noted. The only significant findings on physical examination were marked pallor, slight icterus of the sclerae, a smooth clean tongue, and malnutrition.

**Laboratory Data**—The peripheral blood revealed the presence of a macrocytic hyperchromic anemia. A bone marrow aspiration was compatible with pernicious anemia or sprue. The chest x-ray, a gastrointestinal series and the electrocardiogram disclosed no abnormalities. The urine showed a 1 plus test for albumin. The blood urea nitrogen was 15 mg, and the blood sugar, 115 mg per 100 milliliters. The value for total blood proteins was 6.7 Gm per 100 cc, with an A/G ratio of 2. The total blood cholesterol was 200 mg per 100 cc of which 30 per cent were esters. The icterus index was 7 and the van den Bergh

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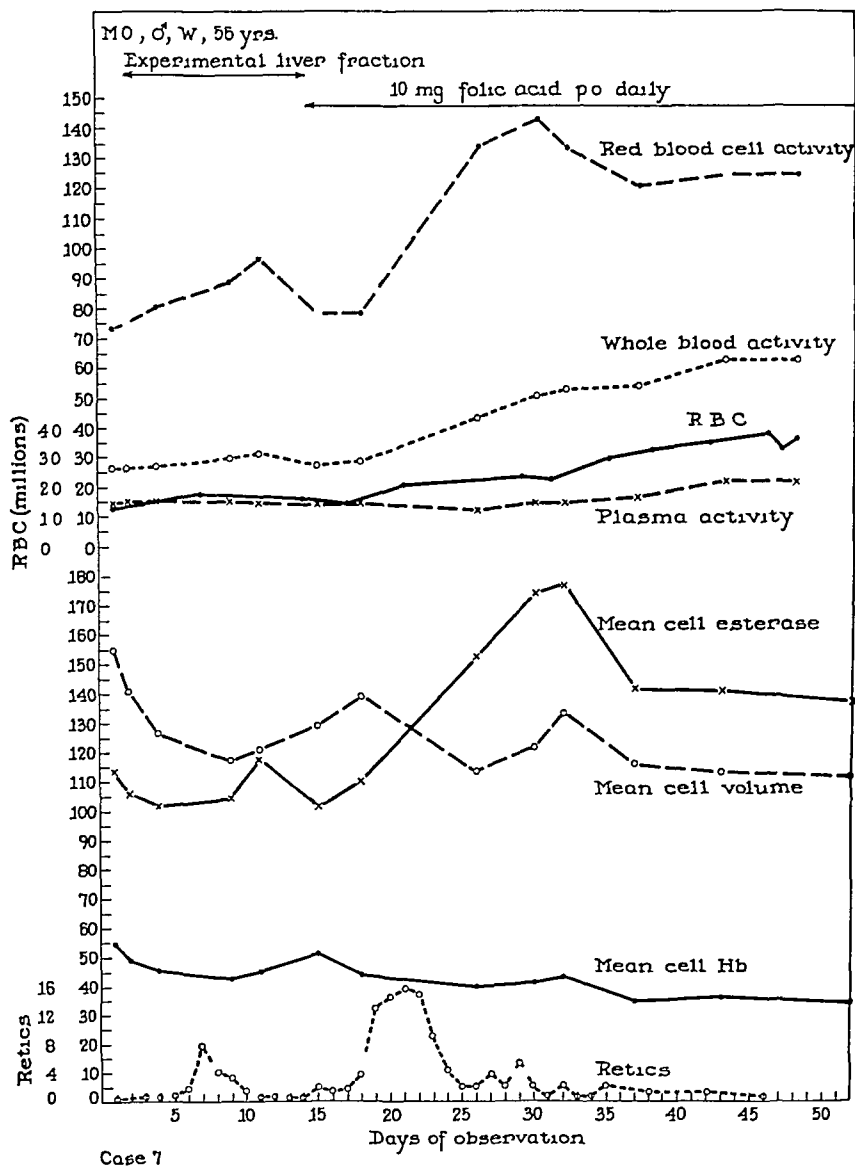


Fig 7

reaction was negative. The cephalin flocculation was negative and the alkaline phosphatase was 19 units.

The patient was treated with a special experimental liver fraction without a clinical response. He was then given 10 mg of folic acid daily by mouth, this treatment was followed by an uneventful clinical and hematologic remission.

Initial red cell and mean corpuscular esterase values were in the normal range. The plasma esterase activity was about one-half the mean normal value. Following the injection of an experimental liver fraction there was definite but unsustained increase in erythrocyte cholinesterase activity. A subnormal reticulocytosis of 7.8 per cent was also noted. There was an equivocal transient rise in the red blood count. When all the values had returned to pretreatment levels, treatment with folic acid in daily oral doses of 10 mg was initiated. This

was followed by a rapid increase in all values except that of plasma cholinesterase, the latter did not change for sixteen days and then rose in a manner paralleling that of the red cell count

## RESULTS

In ten patients with pernicious anemia in relapse the mean esterase activity of the red blood cells was 5.90 ( $\times 10^{10}$ ) units as compared with 7.55 ( $\times 10^{10}$ ) units in normal subjects. In four patients it was within the normal range of 6.19 to 8.69 ( $\times 10^{10}$ ) units. The plasma levels were low with an average activity of 1.60 units as compared with 3.31 units in the normal. Eight of the plasma determinations in the patients with pernicious anemia were within the low normal range (Table I). Three to nine days after beginning effective anti-pernicious anemia therapy there was a rapid rise in red blood cell esterase. The red cell activity rose to above normal value, remained high for thirty-five to eighty-five days, and declined to the average range. The plasma esterase activity remained unchanged at the initial low levels and did not rise until eighteen to thirty days after the onset of effective therapy. The slope of the plasma rise tended to parallel the slope of the erythrocyte rise as both returned to normal.

TABLE I. PERNICIOUS ANEMIA IN RELAPSE

SAMPLE	WHOLE BLOOD CHE (UNITS/ML.)	PLASMA CHE (UNITS/ML.)	RBC CHE (UNITS/ML.)	HB (GM PER 100 ML.)	RBC ( $\times 10^6$ PER MM <sup>3</sup> )	MCA (CU $\mu$ )	CELL CHE (UNITS $\times 10^{-10}$ PER CELL)
M F	2.00	2.32	3.35	6.4	1.68	101	3.38
M P	1.84	1.44	4.29	4.0	1.10	127	5.44
Ja	2.28	1.81	4.75	4.9	1.29	124	5.90
Ve	3.12	2.00	8.59	5.5	1.75	97	8.33
G M	1.82	1.48	4.87	4.0	1.06	122.5	5.96
Kw	1.11	0.54	6.20	3.4	0.76	132	8.15
Kw	1.35	0.98	4.36	6.5	1.30	85	3.69
Cu	1.60	1.14	5.70	3.0	0.72	137	7.90
A I	3.45	2.97	5.61	5.5	1.80	100	5.61
M O	2.64	1.45	7.35	7.0	1.29	155	11.39
Fl.	2.62	1.50	5.81	7.0	2.55	102	5.93
Mean	2.28	1.48	5.61	5.50	1.29	122.5	5.90

Whole blood ChE cholinesterase activity in 1 ml of whole blood. Plasma ChE cholinesterase activity in 1 ml of plasma. RBC ChE cholinesterase activity in 1 ml of red blood cells. MCA mean corpuscular volume in cubic microns. Cell ChE mean corpuscular esterase activity in units per cell ( $\times 10^9$ ).

There was no correlation between the initial red cell esterase activity, the red cell count, or the hemoglobin concentration. The mean corpuscular esterase was similarly unrelated to the mean corpuscular volume. There was an apparent relationship between the initial plasma esterase and the patient's general condition. The plasma esterase was not dependent, however, upon the initial red cell count or the hemoglobin concentration.

Following adequate antipernicious anemia therapy and in patients maintained on adequate therapy, the mean red cell enzyme activity was 8.31 ( $\times 10^{10}$ ) units (normal, 7.55 [ $\times 10^{10}$ ]). The plasma level in these patients was 2.63 as compared with 3.31 in normal subjects. The mean hemoglobin concentration was 14.0 Gm per 100 ml of blood, and the mean erythrocyte count was 4,600,000 per cmm (Table II).

TABLE II PERNICIOUS ANEMIA IN REMISSION

SAMPLE	WHOLE BLOOD ChE (UNITS/ ML)	PLASMA ChE (UNITS/ ML)	R B C ChE (UNITS/ ML)	HB (GM PER 100 ML)	P B C ( $\times 10^6$ PER MM <sup>3</sup> )	M C V (CU $\mu$ )	CELL ChE (UNITS $\times 10^{-10}$ PER CELL)
M F	4 80	2 30	8 00	14 5	4 55	96 5	7 73
M P	5 58	2 80	9 26	13 3	4 80	89 5	8 31
J <sub>1</sub>	5 00	2 10	9 35	14 0	4 60	87	8 23
Ve	5 37	3 43	8 28	15 1	5 20	77	6 37
G M	4 64	1 48	12 00	10 5	3 60	83 5	10 00
Ham	6 18	3 91	9 31	14 0	4 60	91 5	8 56
J A	4 92	2 66	9 11	12 2	4 40	79 5	7 25
A P	5 80	2 86	9 70	14 5	4 90	87 7	8 51
M O	6 30	2 16	12 50	12 0	3 60	111	13 70
Mean	5 37	2 66	9 31	14 0	4 60	87 7	8 31

Whole blood ChE cholinesterase activity in 1 ml of whole blood Plasma ChE cholinesterase activity in 1 ml of plasma R B C ChE cholinesterase activity in 1 ml of red blood cells M C V mean corpuscular volume in cubic microns Cell ChE mean corpuscular esterase activity in units per cell ( $\times 10^{-10}$ )

Four patients (Cases 3, 5, 6, and 7) were given preliminary suboptimal therapy with either folic acid or experimental liver fractions of varying potency without causing a significant rise in the erythrocyte counts. In three of these patients (Cases 3, 5, and 7) small reticulocyte peaks of less than 8 per cent were observed nine, five, and six days, respectively, after injection of the extract. A rapid unsustained peak to normal values of the mean cell esterase was noted nine, four, and ten days, respectively, after the fraction was given. One patient (Case 6) had no reticulocytosis, and no effect on cholinesterase values was seen.

#### DISCUSSION

It would appear that in the normal individual the cholinesterase activity of the whole blood, red cells, and plasma remains constant within a narrow range from day to day and month to month.<sup>15</sup> Patients with pernicious anemia in relapse exhibit low whole blood, red cell, and plasma esterase activity. When therapy is instituted the esterase activity of whole blood and red cells increases rapidly. This increase is shown to be due to an increase in the mean red cell esterase activity and does not reflect an erythrocyte rise which it invariably precedes. The plasma cholinesterase remains constant during this period. In four patients (Cases 1, 2, 3, and 7) the initial rapid red cell cholinesterase rise occurred at the same time that a reticulocytosis was noted. In cases 4 and 6 the reticulocytosis preceded the red cell esterase rise. In Case 5 the reticulocytosis continued even when the red cell esterase activity was falling. Where subeffective therapy was given, minimal reticulocytic response was associated with significant red cell esterase rise. Thus, a direct relationship between red cell esterase and reticulocytosis could not be established. In Cases 2, 3, 5, 6, and 7 the cell cholinesterase was not dependent on the mean cell volume. In two patients (Cases 1 and 4) the red cell esterase curves closely followed the curve of the mean cell volume.

Apparently patients with pernicious anemia in relapse have erythrocytes that are abnormally low in cholinesterase activity, and plasma that exhibits a subnormal esterase activity. The administration of liver extract or folic acid,

parenterally or orally, is immediately followed by an increase in the cholinesterase activity of the red cells. This increase is most frequently carried to levels greater than those found in the red cells of normal subjects. Following a variable period of time (about five to ten weeks) the red cell activity declines to and remains within the normal range. At this time a rise in plasma esterase is noted.

Sabine<sup>1</sup> postulated a normal mechanism for maintaining a high esterase in the mature cell of the anemic patient and the failure of the mechanism in the patient whose marrow is incapable of responding. She further postulated a specific defect in pernicious anemia with an "inability to put out cholinesterase in adequate amounts in such cells as the marrow produces." Davis<sup>10, 11</sup> believes that relapse in pernicious anemia is related to the increased activity of acetyl choline. He believes that this increased concentration produces a dilatation of the vascular network of the bone marrow with an increase in marrow oxygen tension. With this increase in oxygen tension an inhibition of the maturation of cells of the erythroid series occurs.

We have accumulated data<sup>12</sup> in various other anemias. It is our experience that pernicious anemia alone shows the consistently low cholinesterase activities we have described, with the possible exception of myeloid leucemia. Other anemias with similar low hemoglobin and erythrocyte counts are associated with normal or above normal red cell esterase activity. The plasma activity in these cases is dependent upon the patients' general condition being as low as or lower than the levels herein reported. Reticulocyte counts in these cases have revealed no consistent trend.

#### SUMMARY

Observations on seven patients with pernicious anemia in relapse are reported. The cholinesterase activity of whole blood red cells and plasma has been followed during their return to remission.

The cholinesterase activity of whole blood, red cells, and plasma in pernicious anemia patients in relapse is below normal levels.

With therapy, the cholinesterase activity of the red cell is the first to rise. This rise in activity is not related to the reticulocytosis, the mean cell volume, or the increase in the number of red cells.

The plasma activity remains constant until the values for red cell esterase have become abnormally high and then have returned to normal levels.

The authors express their appreciation to Dr. Robert A. Lehman, Department of Therapeutics, New York University College of Medicine, for his many helpful criticisms and suggestion.

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## A STUDY OF CHOLINESTERASE ACTIVITY IN THE BLOOD OF NORMAL SUBJECTS

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DALL,<sup>1</sup> in 1914, postulated the presence of a blood esterase which would inactivate acetylcholine. Loewi, working with the isolated frog heart, demonstrated that stimulation of the vagus liberated a substance which itself was capable of producing the effect previously thought to be due to action of the nerve. Loewi and Naviatil<sup>2</sup> showed that this vagus substance was an ester of choline, probably acetylcholine. These authors also found that aqueous extracts of frog heart inactivated acetylcholine. Because these extracts acted in an enzyme-like manner, they called the active principle an esterase. In 1930 Engelhart and Loewi<sup>3</sup> and, independently, Matthes<sup>4</sup> conclusively demonstrated the enzymic nature of the destructive agent. Three years later, Stedman, Stedman, and Lisson<sup>5</sup> presented evidence of the presence of cholinesterase in the blood serum of the horse. In 1935, Stedman and Stedman reported that sera from different species differed widely with respect to the content of cholinesterase. These authors found that the erythrocytes of various species of animals also contained appreciable amounts of enzyme. By this time a host of papers had appeared and various phases and characteristics of the enzyme were reported in the literature. Almost all of the earlier work was based on the enzyme present in serum or plasma.

Plattner and Galehi,<sup>6</sup> using a biologic assay method, found that the cholinesterase activity of erythrocytes was greater than that of serum. Matthes confirmed this finding. Stedman and Stedman showed in a series of experiments using a physicochemical technique that sera of different species differed widely with respect to the cholinesterase content and that the erythrocytes of various species contained appreciable amounts of enzyme. Hall and Lucas<sup>7</sup> studied normal and pathologic human sera and concluded that age, sex, activity, diet, heart rate, and blood pressure were without influence on the serum esterase. They reported that the range of serum esterase values for any individual was constant, but that this might vary widely from individual to individual. In 1940, Sabine<sup>8</sup> showed that the cholinesterase activity of erythrocytes was greater than that of plasma in both normal and pathologic subjects. Allen and Hawes<sup>11</sup> confirmed her findings.

Brauer and Root<sup>9</sup> recently demonstrated a correlation between liver cholinesterase and the serum esterase of the dog. Similarly it has been shown<sup>13</sup> that the rate of human serum esterase regeneration is significantly lower in patients with liver disease than in the normal subject. Cohn and co-workers<sup>14</sup> have found that plasma esterase activity is largely concentrated in plasma fraction IV<sub>4</sub>. This plasma fraction is rich in alpha and beta globulins of low lipid

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content Mendel and associates,<sup>15, 16</sup> on the basis of substrate concentrations and substrate specificity, postulate two different cholinesterases in the body—one a specific of true esterase found in red blood cells and brain tissue, the other a nonspecific or pseudoesesterase found in serum and plasma Nachmansohn and Rothenberg<sup>17, 18</sup> have inclined toward the view that specificity is relative and that tissues containing the specific esterase split acetylcholine at a higher rate than they split other esters Bodansky<sup>19</sup> speaks of a "family" of cholinesterases whose members resemble one another in some characteristics and differ in others Thus, within one species the enzyme differs in certain respects from tissue to tissue, and in a given tissue, from species to species

#### METHODS

The plasma and red cell cholinesterase activities of random blood samples taken from nine male and six female subjects were determined Serial samples were tested at monthly intervals in three of these subjects Oxalated venous blood was drawn from a vein in the antecubital fossa The hematocrit (Wintrobe) was determined and the samples separated into whole blood and plasma A complete capillary blood count was done at this time Random reticulocyte counts were done in five subjects

The cholinesterase activity was measured in units representing the number of milliliters of 0.02N sodium hydroxide necessary to neutralize the acetic acid liberated from 100 ml of 0.012M acetylcholine bromide solution by 1 ml of whole blood or plasma in a twenty minute period at a pH of  $7.4 \pm 0.05$  The temperature was held constant at  $37.5^\circ \text{C}$  Red cell esterase activity was calculated from the whole blood activity, plasma activity, and the hematocrit value A correction factor applied to all esterase values was derived from a determination of the amount of acetylcholine hydrolyzed under the same conditions in the absence of blood The mean corpuscular esterase was calculated by multiplying the red cell esterase activity by the mean corpuscular volume

$$\text{Mean cell ChE} = \text{Red Cell ChE} \times \text{MCV}$$

$$\text{Units/cell} = \text{Units/ml} \times \text{ml/cell}$$

#### RESULTS (SEE TABLES I AND II)

TABLE I CHOLINESTERASE ACTIVITY OF NORMAL HUMAN SUBJECTS

SAMPLE	WHOLE BLOOD CHE (UNITS/ ML)	PLASMA CHE (UNITS/ ML)	R B C CHE (UNITS/ ML)	HB (GM PER 100 ML)	R B C ( $\times 10^6$ PER MM <sup>3</sup> )	MCV (CU $\mu$ )	C CHE (UNITS $\times$ $10^{-10}$ PER CELL)
A S ♂	6.06	3.51	9.44	15.5	5.25	82	7.74
B S ♀	4.46	1.50	8.55	14.6	5.05	84	7.19
L E ♀	5.33	2.71	9.10	15.9	5.15	80	7.28
H R ♂	6.71	3.33	10.34	16.1	5.75	84	8.69
B F ♂	5.14	2.58	8.40	15.2	5.25	84	7.06
A J K ♂	5.22	1.83	8.90	16.2	5.30	91	8.10
C R ♂	6.62	4.14	9.64	14.3	5.10	88	8.47
J M ♀	5.39	3.95	7.44	14.0	4.85	85	6.32
F K ♀	6.65	4.92	8.76	15.4	5.20	87	7.64
A O ♂	6.84	4.13	10.16	17.0	5.65	80	8.12
B Sch ♂	4.83	2.92	7.25	14.1	5.16	85	6.19
L G ♀	6.76	5.00	8.52	16.2	5.30	94	8.06
P S ♀	5.08	3.00	7.84	15.0	4.85	89.5	7.02
S S ♂	5.40	2.95	8.40	15.1	5.20	86.5	7.27
H K ♂	5.81	3.19	8.82	15.5	5.42	85	7.55
Mean	5.40	3.19	8.76	15.4	5.20	85	7.55

Whole blood ChE cholinesterase activity of 1 ml whole blood Plasma ChE cholinesterase activity of 1 ml plasma R B C ChE cholinesterase activity of 1 ml red blood cells MCV mean corpuscular volume C ChE mean corpuscular cholinesterase activity, units per cell

TABLE II CHOLINESTERASE ACTIVITY OF NORMAL HUMAN SUBJECTS, REPEATED SAMPLINGS

SUBJECT	DATE	WHOLE BLOOD CHE (UNITS/ML)	PLASMA CHE (UNITS/ML)	RBC CHE (UNITS/ML)	CHE (UNITS/CELL)
A S	12/9	6 13	3 43	9 42	7 76
♂	2/5	6 06	3 51	9 44	7 74
	4/7	5 98	3 20	9 38	7 47
A J A	12/9	5 22	1 83	8 90	8 10
♂	2/24	5 40	2 02	9 06	8 15
	5/23	5 30	1 97	9 06	8 05
S S	2/5	5 40	2 95	8 40	7 27
♂	4/7	5 52	3 01	8 46	7 26
	5/23	5 46	2 89	8 60	7 54

The cholinesterase activity of 1 ml of red blood cells ranged from 7.25 to 10.34 units, with a mean value of 8.76 units. The mean corpuscular esterase activity varied from 6.19 to 8.69 units ( $\times 10^{10}$ ), with a mean value of 7.55 units ( $\times 10^{10}$ ). The esterase activity of 1 ml of plasma was found to be 1.50 to 5.00 units, with a mean of 3.19 units. Activity did not seem to be influenced by sex. Although the activity varied markedly from individual to individual, within the same subject the activity remained rather constant (see Table II).

## SUMMARY

Our results confirm the findings of previous workers that in the human subject cholinesterase activity of the red blood cell is greater than that of the plasma. The cholinesterase activity may vary widely from individual to individual, but tends to remain fairly constant in any one individual.

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# A COMPARISON OF METHODS USED FOR THE HYDROLYSIS OF CONJUGATED URINARY ESTROGENS

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A PREVIOUS report from this laboratory suggested the need for a thorough study of the methods for the hydrolysis of urinary estrogens. Gallagher<sup>7</sup> has reviewed a number of the factors concerned in urine hydrolysis and has pointed out that present methods are not entirely satisfactory.

In addition, the fact that most methods of urine hydrolysis have established definite time intervals of treatment indicates that acid hydrolysis of urine may be, in reality, a balance between two simultaneous reactions namely, hydrolysis and destruction. The method giving the greatest yield of free forms, then, would be one which caused the hydrolysis of a maximum amount of conjugated forms while the destructive process was kept at a minimum. The mechanics of the reactions that cause a decrease in biologic activity of estrogen preparations subjected to acid hydrolysis are as yet unknown. Pincus and Pearlman<sup>11</sup> have reviewed the subject of artifacts arising from the acid treatment of androgen preparations and they point out the misconceptions that may arise from such procedures.

Good yields of free estrogens were obtained by Cohen and Marrian by adjusting the pH of the urine to 1.0 with HCl, further acidifying with 3.3 ml of 12 N HCl per 100 ml urine, and finally, heating the sample at 120° C. for two hours. Smith and Smith<sup>1</sup> advocated the use of reflux hydrolysis for ten minutes of urine samples made to 15 volumes per cent with HCl and found good agreement between their method and the Cohen and Marrian procedure. Dingemans, Laqueur, and Muhlbock<sup>4</sup> hydrolyzed urine with 15 ml of 25 per cent HCl per 100 ml urine and at the same time extracted freed forms with a layer of benzene. Three four hour periods of boiling with a total of 150 ml of organic solvent were used.

Other methods of treatment have been suggested<sup>3, 8, 9, 13</sup> but because of difficulties of too mild or too drastic conditions have not been adopted generally.

Leiboff and Tamis,<sup>9</sup> Smith and Smith<sup>12</sup> and Gallagher, Peterson, Dorfman, Kenyon, and Koch<sup>6</sup> have used continuous extraction equipment for the extraction of hydrolyzed urine. Talbot, Butler, MacLachlan, and Jones<sup>15</sup> reported a procedure for the simultaneous hydrolysis and extraction of urinary 17 ketosteroids.

Before urine hydrolysis studies were undertaken the effect of hydrolysis conditions on pure estrogens was ascertained.

This work is admittedly incomplete but since it is not possible to continue the investigation at this time these results are being published in the hope that they may be of interest to investigators in the field.

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*Effect of Acid Hydrolysis on Free Estrogens*—Smith and Smith<sup>14</sup> found there was no loss of activity of estione, estriol, or  $\alpha$ -estradiol when solutions of these forms were treated by their hydrolysis conditions, namely 100° C for ten minutes with 15 volumes per cent HCl, but they found there was an appreciable destruction of estione by heating at 100° C for twenty minutes with 15 volumes per cent HCl

We have confirmed the observation that prolonged boiling of pure estrogens in 15 volumes per cent HCl causes destruction. Thirty minutes of boiling of pure estrogens in distilled water made 16 N with HCl caused 28 per cent destruction of estriol, 34 per cent of estione, and 34 per cent of  $\alpha$ -estradiol. As previously reported,<sup>5</sup> no destruction of activity occurred when pure estrogens were hydrolyzed in an atmosphere of nitrogen and in the presence of a protective agent (1-amino-2-naphthol-4-sulfonic acid). Residual estrogenic activity was determined by bio-assay, for colorimetric determinations on these same fractions showed a wide discrepancy from bio-assay results.

*Hydrolysis of Known Conjugated Estrogen*—The principal estrogen conjugate in human urine appears to be estriol glucuronide. Although estrone also may be excreted in a conjugated form (perhaps as the sulfate, as in mare urine), such a compound has not been isolated from human sources.

The conditions affecting hydrolysis of the pure estriol conjugate have not been reported previously. We have studied the behavior of this compound under three conditions listed below. Aliquots of standard solutions of sodium estriol glucuronate\* were added to distilled water and further treated in the following manner. The first sample was adjusted to a pH of 1.0 with HCl and allowed to stand at room temperature for ten days. Such treatment liberated only 5 per cent of the combined form. The second sample was hydrolyzed for ten minutes at 100° C with 15 volumes per cent HCl, under N<sub>2</sub> and in the presence of the previously described protective agent, and gave 99 per cent hydrolysis. The third preparation was treated like the second except that only enough HCl was added to make the solution 0.1 N. In this case only 20 per cent of the estriol was liberated.

The second and third samples show the importance of acid concentration and the necessity of heat treatment and also indicate that the acid and heat treatment of the Smith and Smith<sup>12</sup> procedure are effective hydrolytic agents for this conjugate in pure solution.

*Hydrolysis of Pregnancy Urine*—Preliminary studies on each of five urines obtained during the fifth and sixth month of pregnancy indicated a considerable variation in results. The Smith hydrolysis and our protective hydrolysis modification of the Smith method were the chief methods employed. These studies indicated that nitrogen and the protective agent in a few cases gave higher amounts of freed estrogens. As Doisy has previously reported,<sup>6</sup> in one of the urines tried, the potency of an aliquot of a twenty-four hour specimen was doubled as the time of hydrolysis was increased from ten minutes to forty-five minutes.

\*Obtained through the kindness of A. S. Cook of Averst McKenna and Harrison Montreal, Canada.

To minimize the possible variations given by single isolated specimens and to insure an adequate supply of urine for future work an 18 liter sample of pregnancy urine was collected by pooling twenty four hour urine specimens from patients seven months pregnant. This pooled urine was preserved with chloroform, the pH adjusted to 7.0, and the urine filtered and stored at 3 to 5° C. The experiments to be described represent a comparison of a number of techniques all carried out on aliquots of this stool urine.

Bio assay, by a modified Marrian and Parker method recently described by Thayer, Doisy, Jr., and Doisy<sup>16</sup> was conducted on all estrogenic fractions.

After preliminary assays had determined the proper dose level, the final assays of an experiment were run on a group of animals available on a single assay day. A minimum of twenty animals was used at each assay level and twenty animals served as controls. Although great care was taken in these bio assays it must be kept in mind that differences in results of 20 per cent probably are not significant.

The yield of estrogen obtained in each of the experiments indicated that although ether extracts of hydrolyzed urines were further separated into phenolic, nonphenolic, and acidic fractions, colorimetric analysis by a modified sulfanilic acid method (Van Bruggen<sup>17</sup>) and by Bachman's method<sup>1</sup> gave results that differed markedly from the bio assay results. Although a few such chemical determinations gave good agreement with bio assay figures, for the most part the colorimetric determinations gave values from two to eight times higher than bio assays.

Preliminary studies indicated that the ten minute hydrolysis with 15 volumes per cent did not give complete hydrolysis of combined forms. To test this possibility, several experiments were carried out.

*Experiments 1 and 2*—Of the 18 liter pooled sample, 1,200 ml. were acidified to 15 volumes per cent with HCl and refluxed in an all glass apparatus fitted with a siphon. This arrangement permitted the withdrawal of samples of the boiling urine at various time intervals. One hundred fifteen milliter samples were withdrawn, cooled under water, and saturated with NaCl to help prevent emulsion formation. The estrogens were extracted from the treated urine with four 50 ml. portions of purified ethyl ether. The combined ether extracts were washed with 9 per cent NaHCO<sub>3</sub>, dilute HCl and distilled water. The ether was removed by distillation and the residue taken up in 95 per cent ethanol. The zero hour sample was taken after acidification but before the system was heated and should thus represent free estrogens present. A similar experiment was carried out but differed from the first in that 125 mg. of 1 amino 2 naphthol 4 sulfonic acid per 100 ml. urine were added to the urine. In addition N was passed through the urine for a ten minute period before heating was started as well as during the hydrolysis. Table I lists the data obtained from these two experiments.

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TABLE I HYDROLYSIS OF POOLED PREGNANCY URINE WITH 15 VOLUMES PER CENT HCl (COLUMN A, ACTIVITY RECOVERED WITHOUT PROTECTION, COLUMN B, ACTIVITY RECOVERED WITH N<sub>2</sub> AND THE PROTECTIVE AGENT)

SAMPLE	TIME OF HYDROLYSIS (MIN)	M U PER LITER	
		A	B
1	0	37,812	34,300
2	10	75,050	98,250
3	20	91,313	98,250
4	30	110,000	75,000
5	40	96,250	80,000
6	50	110,000	98,000
7	60	89,375	121,000
8	90	75,625	60,500
9	120	86,400	70,000
10	240	-----	50,000

1 ml of concentrated HCl was added, to the other two, 3 ml additional HCl were added. One of these two also received a small amount of the protective agent. Two other 100 ml samples were adjusted to a pH of 1.00 with H<sub>2</sub>SO<sub>4</sub>. One of these received an additional 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub>, the other, 3 ml of H<sub>2</sub>SO<sub>4</sub>. The five flasks were then simultaneously autoclaved at 120° C for two hours. After this pressure hydrolysis, the samples were cooled and extracted by the technique mentioned in Experiment 1. Table II compares the results of the experiment on the five samples.

TABLE II TWO HOUR PRESSURE HYDROLYSIS AT 120° C OF ALIQUOTS OF THE POOLED URINE, ALL SAMPLES BEING FIRST ADJUSTED TO pH 1.0 WITH THE APPROPRIATE ACID

SAMPLE	ADDITIONAL TREATMENT	M U PER LITER
1	1 ml HCl per 100 ml urine	116,880
2	3 ml HCl per 100 ml urine	123,750
3	3 ml HCl per 100 ml urine plus protective agent	132,000
4	1 ml H <sub>2</sub> SO <sub>4</sub> per 100 ml urine	86,663
5	3 ml H <sub>2</sub> SO <sub>4</sub> per 100 ml urine	103,120

*Experiment 4*—The high activity of fractions 4-A and 7-B of Experiments 1 and 2 (Table I) after the ten-minute time as well as the good yield of the third fraction (Table III) suggested that the ten-minute hydrolysis with 15 volumes per cent HCl was not liberating all of the combined estrogens. This experiment was devised to determine the degree of hydrolysis effected by the Smith<sup>12</sup> method as performed in our experiments. One liter of the pooled urine was made acid to 15 volumes per cent with HCl and heated in the same apparatus used in Experiments 1 and 2. Samples were taken and extracted four

TABLE III PRELIMINARY HYDROLYSIS AND EXTRACTION (COLUMN A) AND SUBSEQUENT RECOVERY OF ESTROGENS BY HYDROLYSIS AND EXTRACTION (COLUMN B)

SAMPLE	TIME OF HYDROLYSIS OF FIRST SAMPLE (A)	M U PER LITER		TOTAL RECOVERY (A PLUS B)
		A	B	
1	0	38,800	55,000	93,000
2	10	95,000	31,250	126,250
3	30	82,500	6,250	88,750
4	60	110,000	800	110,800
5	90	82,500	1,000	83,500
6	120	89,375	1,200	90,575

times with ether as before. However, after free estrogens had been extracted with ether, the urine residue was thoroughly extracted with butyl alcohol to remove conjugated forms. The butyl alcohol was concentrated, the residue dissolved in 100 ml water, HCl was added to 15 volumes per cent, and the extract hydrolyzed for ten minutes, the estrogens were removed with ether as before. Each extract was assayed separately. In Table III, column A represents the activity obtained by the first hydrolysis and column B represents activity present in the hydrolyzate of the butanol extractions. Activity in B, then, represents estrogens not hydrolyzed or extracted by a simple acid hydrolysis.

*Experiment 5*—The high activity of the B fraction of the ten minute hydrolysis of the previous experiment might be due to one or both of two factors. Although the distribution coefficient of an acid ether partition strongly favors the movement of estrogens into the organic phase, it is possible that faulty extraction of the urine might account for the activity of the B fraction. On the other hand, incomplete hydrolysis at ten minutes might cause the retention of activity in the water phase. The following data were obtained in an effort to clear up this matter. An aliquot of the pooled urine was hydrolyzed for ten minutes with 15 volumes per cent HCl. After the customary ether extraction, the urine residue was extracted with butanol. The butanol extract was divided into two equal parts, one half of which was concentrated at a low temperature under suction to avoid hydrolysis during this time. The other half of the butanol extract was concentrated and rehydrolyzed as in Experiment 3. This fraction then contains the ether soluble estrogens after hydrolysis of the butanol extract. In the original ether extract 103,100 M U per liter were found, 6,000 M U per liter were found in the butanol extract that was not rehydrolyzed. The butanol fraction that was rehydrolyzed showed 15,000 M U per liter.

The effectiveness of continuous extraction and hydrolysis was studied in a number of experiments. It was felt that if the free estrogens could be extracted by an organic solvent immediately after their release from the conjugate form, that destruction might be minimized. Attempts to accomplish this by a number of methods are reported below.

*Experiment 6*—One hundred milliliter samples of the pooled urine were made to 15 volumes per cent HCl and were then layered with 100 ml amounts of toluene, butanol, benzene, and cyclohexanol, respectively. Such systems were boiled under reflux for two hours. During the heating period, good contact between the urine and the organic solvent was assured by the bubbling of a stream of nitrogen through the solutions. After the two hour heating the urine and solvent were cooled the organic solvent layer removed, and the urine residue further extracted with three additional portions of the same solvent used during the heating period. Toluene gave a recovery of 41,300 M U per liter, butanol, 82,500, benzene, 64,800, and cyclohexanol, 70,000.

*Experiment 7*—Since the original hydrolysis and extraction described by Dingemans and co workers<sup>4</sup> was for a considerably longer period of time, and used, in addition, a greater total amount of solvent, the procedure of Experiment 6 was not comparable. To approximate the conditions used by Dunge

manse, 100 ml of urine were acidified with 23 ml HCl, the urine covered with 100 ml benzene, and the mixture refluxed for four hours. The benzene was removed and replaced with 100 ml fresh solvent and the urine and benzene again refluxed. A third such treatment gave a total of 300 ml benzene used during a total hydrolysis and extraction period of twelve hours. The benzene solutions were combined, washed with a small amount of water, and taken to dryness. By this method, a total of 500,000 M U per liter was obtained.

*Experiment 8*—The principle of the previously mentioned continuous hydrolysis and extraction procedure of Talbot and associates<sup>15</sup> was applied to our problem of estrogen recovery. The apparatus used was similar in construction to that described by Smith and Smith<sup>12</sup>. The lighter-than-water solvents used were delivered to the bottom of the urine phase by a tube having a porous glass disk at the bottom. The column of urine was surrounded, in addition, by a water bath that permitted the maintenance of the urine at elevated temperatures. With benzene as the circulating solvent, the urine was kept at 70° C and 77,000 M U per liter were obtained, with toluene the temperature was 80° C, 77,000 M U per liter being found, and cyclohexanol permitted a temperature of 90° C with a recovery of 100,000 M U per liter.

TABLE IV HYDROLYSIS OF CONJUGATED ESTROGEN IN BUTYL ALCOHOL

SAMPLE	TIME OF HEATING	M U PER LITER
1	0	74,250
2	10	107,250
3	60	103,125
4	120	148,500

*Experiment 9*—Liquid ammonia has within recent years been extensively employed in organic hydrolysis procedures. Hydrolysis with liquid ammonia (ammonolysis) was attempted in two ways. In Experiment 9a the total estrogens were extracted from urine with butanol. After the butanol was evaporated, the residue was taken up in 20 ml dry butanol and 40 ml liquid ammonia were added. The mixture (in an open glass tube) was enclosed in a bomb made of two-inch steel pipe capped at each end by ordinary pipe caps. After forty-eight hours at room temperature the residue was distilled to dryness and taken up in 95 per cent ethanol as usual. In Experiment 9b, a butanol extract of urine was taken to dryness, the residue transferred to the glass inner tube of the bomb, and the dry residue treated with liquid ammonia. A total of 48,125 M U per liter was obtained by the first treatment and 68,750 M U were obtained by the second method.

*Experiment 10*—It was thought that if the total estrogens could be removed from the urine and the combined forms then hydrolyzed, that destruction might be reduced. To this end, 500 ml of the pooled urine were made acid to congo red and extracted with five 100 ml portions of butyl alcohol. The combined butanol extracts were washed once with water, made to 15 volumes per cent with HCl and hydrolyzed as in Experiment 1. One hundred thirty milliliter samples were withdrawn (equivalent to 100 ml urine), cooled,

and concentrated alkali added until the aqueous phase which separated was just acid to litmus. The alcohol was concentrated and the residue taken up in ethanol.

Table IV gives the results of the previously described experiments in terms of mouse units per liter of urine.

#### DISCUSSION

In discussing the results of these experiments it should be kept in mind that the yields by the various techniques have been obtained by bio assay and that differences of 20 per cent are not significant. Experiment 1 bears out the previous suggestion that at least with the pooled urine used in this work a ten minute hydrolysis with 15 volumes per cent HCl does not hydrolyze all the conjugated forms present. Since boiling for periods greater than twenty to thirty minutes in 1.6 N HCl is known to destroy estrogens it seems likely that the high activity present at forty minutes is due to the presence of a hydrolytic process and its predominance over a destructive process. There seems to be little advantage to using nitrobenzene and the sulfonic acid as in Experiment 2 although experiments on pure estrogens had demonstrated the effective protection of such treatment.

The data of Experiment 3 indicate that pressure hydrolysis is an effective method for urine hydrolysis. Protection of labile forms by the mild reducing agent was of definite value in this procedure. The good recovery of activity by the pressure hydrolysis method recommends it for a routine procedure.

Experiment 4 bears out the previous contention that in this work the ten minute hydrolysis with 15 volumes per cent HCl does not give a quantitative yield of estrogens. The total of 93,000 units of the zero hour sample closely approximates the 95,000 units given by the regular ten minute hydrolysis of the ten minute sample. The 31,250 units of the rehydrolyzed specimen of the ten minute sample, however, represent approximately a 25 per cent potential loss when the routine Smith<sup>1</sup> method is used. The activity of the rehydrolyzed specimens even up to 120 minutes might indicate the presence of some difficult hydrolyzable or extractable form in pregnancy urine.

That the usual four extractions with ether may not remove all the supposedly ether soluble forms is indicated by Experiment 5. The 6,000 units not extracted by the ether represent 5 per cent of the total activity potentially lost while the sum of the second and third fractions (21,000 units) represents a 17 per cent over all loss.

The protection offered by the layering used in Experiment 6 does not appear to have been outstanding. It is possible that better yields might have been obtained if shorter periods of heating had been used. In these four samples the good yield by the use of butanol is in agreement with the results obtained in Experiment 10.

The low recovery of activity seen in Experiment 7 is probably due to the excessively long heating period used. The acid concentration used was somewhat greater than that recommended by Dingemans<sup>4</sup> so that the method is not exactly as described by that author.

The use of continuous hydrolysis and extraction as in Experiment 8 seems to be of promise, however, the high boiling point ( $161.5^{\circ}\text{C}$ ) of the cyclohexanol makes it undesirable as a routine solvent. Somewhat different periods of heating and temperature conditions might greatly improve this procedure.

Liquid ammonia as used in Experiment 9 did not cause appreciable hydrolysis of conjugated forms. A more refined bomb container which would allow the retention of higher pressure might greatly enhance the utility of this method.

The use of butanol as the solvent and hydrolyzing medium, as in the final experiment, appears to be of definite promise. Woolf, Viergiver, and Allen<sup>18</sup> have shown that the method of extraction used in this experiment would quantitatively extract all the pregnandiol glucuronide so that it is probable that most of the estriol complex was removed. The 74,000 units of the zero hour sample probably represent some hydrolysis of conjugated forms during the removal of the butyl alcohol from the sample. The 148,000 units present in the two-hour fraction were the highest yield obtained in these studies.

#### SUMMARY

A number of factors affecting the hydrolysis and extraction of urinary estrogens have been investigated. The use of 15 volumes per cent HCl for ten minutes as the hydrolyzing medium, although giving reasonably good results, does not appear to give optimum yields. Pressure hydrolysis under conditions similar to those advocated by Cohen and Marrian<sup>2</sup> is of definite advantage. The hydrolysis of combined forms in an organic solvent (butanol) gave the highest yields obtained. Preliminary work indicated that there might be wide differences in individual samples of urine, the use of a pooled sample presumably nullified these individual differences. It is possible that if studies such as have been presented in this paper were complemented by a fractional analysis of all urine samples, considerable light could be thrown on the hydrolysis and destructive reactions.

The author gratefully acknowledges the help of the staff of the Department of Biochemistry and especially the services of Miss Corinne Dewes.

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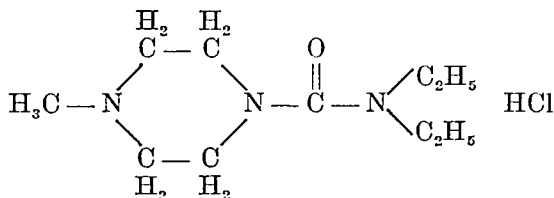
# STUDIES ON THE CHEMOTHERAPY OF FILARIASIS

## VI SOME PHARMACODYNAMIC PROPERTIES OF 1-DIETHYLCARBAMYL-4-METHYLPYPERAZINE HYDROCHLORIDE, HETRAZAN

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THE introduction by Hewitt and co-workers<sup>1, 2</sup> of 1-diethylcarbamy-4-methyl piperazine hydrochloride as a filaricide effective in experimental animals and in man has necessitated an investigation of the pharmacologic properties of this compound. The use of piperazines in medicine is not new. For more than a decade prior to 1918<sup>3</sup> piperazine and 3,6-dimethylpiperazine were accepted remedies for the treatment of gout and rheumatism. Although these drugs were worthless in these diseases,<sup>3, 4</sup> the years of use served to establish the low toxicity of the nucleus and to provide excellent descriptions of the symptoms of over dosage.

1-Diethylcarbamy-4-methylpiperazine hydrochloride, also referred to as Hetrazan and 84-L, has a molecular weight of 234.6<sup>5</sup> and has the following structural formula:



It is a colorless crystalline solid, highly soluble in water, alcohol, and chloroform, but insoluble in benzene, ether, and petroleum ether. The pH of a 10 per cent solution is 4.1. In the pharmacologic experiments the solutions were adjusted to pH 7.4.

### METHODS

The acute toxicity of single doses was studied in six species. Multiple doses at short intervals were given to mice, rats, and dogs. Studies on the chronic toxicity were made with rats, rabbits, dogs, and chickens.

Tests for irritation were made by intracutaneous injections in guinea pigs of 0.1 cc of 10 per cent solutions and by local application of the same concentration to the eyes of cats. Antihistaminic action was tested on the isolated guinea pig gut and on guinea pigs in a spray chamber. Cats were used for pupillary studies. Hemoglobin was determined as cyanmethemoglobin.<sup>6</sup> The Lapschutz assay<sup>7</sup> was used to evaluate diuretic action. Analgesia was determined by a method which employed the application of heat to the rat's foot.<sup>8</sup>

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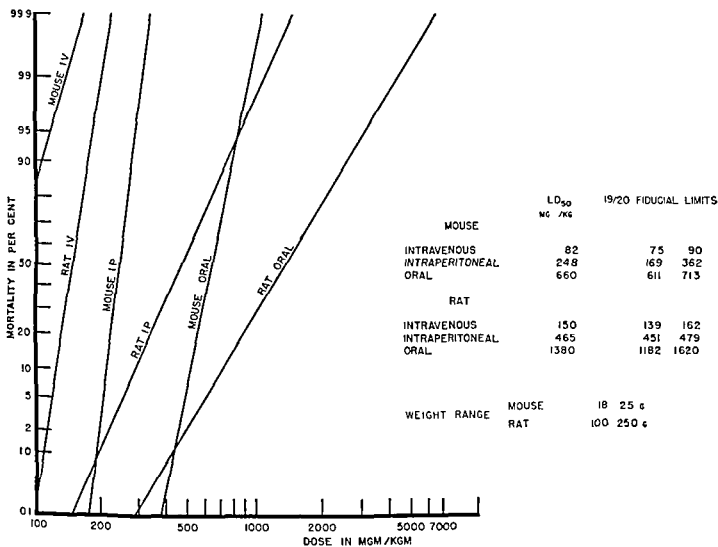
Respiratory movements were relayed to a kymograph by a string attached halfway between the inferior end of the sternum and the umbilicus. Electrocardiographic studies were made with a Cardiotron on anesthetized and unanesthetized dogs. In anesthetized animals blood pressure was recorded from the carotid artery. In unanesthetized dogs the pressures were recorded by femoral arterial puncture.

## RESULTS

## ACUTE TOXICITY —

*Single Doses —*

*Mice and Rats* The dose mortality curves recorded in Fig 1 summarize the data from 238 mice and 260 rats. Toxic doses produced convulsions that were predominately tonic. The convulsant dose however was considerably below the fatal dose.



## ACUTE TOXICITY OF 1-DIETHYL CARBONYL 4-METHYL PIPERAZINE HYDROCHLORIDE (84L)

Fig 1—The experimental results were plotted on log probability paper\* and straight lines were fitted by eye. The 19/20 fiducial zones were estimated by a modification of the method of Litchfield and Fertig.<sup>6</sup> The modification allowed for the fact that the population was not homogeneous in all cases and consisted of correcting the estimated values by multiplying

by  $\sqrt{\frac{(Chi)}{n}}$  as described by Wilcoxon and McCallan.<sup>7</sup>

*Guinea Pigs, Rabbits, Cats, and Dogs* Lethal doses were not determined in these species but large doses were tolerated with few signs of toxicity. Ten guinea pigs given 50 mg per kilogram intraperitoneally showed no changes. Objectionable reactions were not observed in fifteen rabbits given 100 mg per kilogram by the same route. Twenty five milligrams per kilogram given intra



peritoneally to six cats and 50 mg per kilogram given to three cats caused vomiting in six to ten minutes. In addition, these animals showed slight drowsiness.

Serious reactions were not observed in unanesthetized dogs that had received 100 mg per kilogram orally or intraperitoneally or one-fifth of this dose by rapid intravenous injection. If reactions occurred they were, in order of frequency, nausea, vomiting, and muscular tremors. The tremors closely approximated those of a dog shivering from a low temperature. In addition to these reactions, intravenous injections caused stimulation of the respiration which lasted from one to three minutes (Fig 3). Oral doses of 50 mg or more per kilogram often produced emesis, but the presence of food in the stomach decreased the frequency of this occurrence. Oral doses of 25 mg per kilogram administered with food usually were retained.<sup>1</sup> Table I contains the data on thirty-nine dogs.

TABLE I REACTIONS OF UNANESTHETIZED DOGS TO HETRAZAN (84 L)

NUMBER OF DOGS	DOSE (MG /KG )	ROUTE OF ADMINISTRATION	SYMPTOMS			
			NAUSEA	EMESIS	RESPIRATORY STIMULATION	SHIVERING
			INCIDENCE IN PER CENT			
11	5	Intravenous†	9	9	100	0
2	10	Intravenous†	0	0	100	0
4	20	Intravenous†	75	50	100	0
1	25	Intravenous†	100	0	100	100
1	50	Intravenous†	100	100	100	100§
2*	100	Intraperitoneal	100	100		100
5	50	Oral‡	80	60		20
12	100	Oral‡	66	66		8
1	200	Oral‡	100			100

Respiratory stimulation lasted about one minute. Shivering lasted from thirty to sixty minutes. Vomiting never was accompanied by continued retching or signs of malaise. Recovery was excellent in all dogs.

\*These dogs were given 100 mg per kilogram twice daily for two days.

†The intravenous injections were completed within one minute.

‡Dogs were fed two to five hours before dosing.

§Severe muscular tremors.

### Repeated Doses —

Since the antifilarial data of Hewitt and associates<sup>1</sup> indicated the necessity for frequent administration of Hetrazan it seemed desirable to study the rate of its destruction or excretion. For this study experiments were designed to give rats and mice repeated injections at a rate which barely exceeded the capacity of the animal to eliminate the compound. The criteria of accumulation were the incidence of convulsions and the percentage of mortality.

In rats the intraperitoneal LD<sub>50</sub> is 465 mg per kilogram (Fig 1), therefore, in the tolerance studies a single intraperitoneal dose of 300 mg per kilogram was given at zero time, with additional doses of 100 mg per kilogram at hours 2, 3, 4, 5, 6, 7, and 8. The incidence of convulsions provided a delicate indicator of the rate of elimination of the compound (Table II). At 300 mg per kilogram 100 per cent of the animals convulsed, but after an interval of two hours an additional dose of 100 mg per kilogram produced no convulsions.

The incidence of convulsions after the third and fourth doses was 9 per cent in each case and after the eighth dose, 45 per cent. Although the total dose given to these rats amounted to 1,000 mg per kilogram, no increment subsequent to the initial dose produced convulsions in all rats. In three groups of rats, sixty five animals, 300 mg per kilogram never failed to produce convulsions. Thus it appears that under the foregoing conditions the rat is capable of eliminating approximately 100 mg per kilogram per hour.

TABLE II THE EFFECTS ON RATS OF MULTIPLE INTRAPERITONEAL DOSES OF 84 L REPEATED AT SHORT INTERVALS

INTERVAL SUBSEQUENT TO INITIAL DOSE (hours)	NUMBER OF RATS INJECTED	DOSE		INCIDENCE OF CONVULSIONS (%)	MORTALITY (%)
		SINGLE (MG/KG)	CUMULATIVE (MG/KG)		
0	45*	300	Initial dose	100	31.1
	Group I†				
2	22	100	400	0	0
3	22	100	500	9.1	4.1
4	21	100	600	9.5	0
5	21	100	700	19.0	0
6	21	100	800	4.7	0
7	21	100	900	28.6	0
8	11	100	1000	45.5	0‡
	Group II				
4	9	300	600	100	22.2
5	7	150	750	43	0
6	7	150	900	43	0
7	7	150	1050	71.4	42.8‡

Range of weight in grams 200 to 250

\*The survivors were divided into two groups I and II

†Combined results of a group of 10 and a group of 12 rats dosed on different days.

‡Twenty four hours later all animals were in good condition

Obviously the validity of this calculation rests on the assumption that the rat does not become resistant to the convulsant action of the compound. This question has been answered by the data on Group II in Table II. In this series the original dose of 300 mg per kilogram was repeated after four hours. The incidence of convulsions was again 100 per cent and the mortality, 22 per cent. When additional doses of 150 mg per kilogram per hour were given on the fifth, sixth, and seventh hours, the incidence of convulsions rose from 43 per cent on the fifth hour to 71 per cent on the seventh. These data show that rats do not become resistant to the convulsant action of 84 L and also that 150 mg per kilogram per hour exceed the ability of the rat to eliminate the compound.

In Group I, Table II, the mortality from the initial dose of 300 mg per kilogram was 31 per cent, and from the succeeding doses, 700 mg per kilogram in six hours, 5 per cent. A similar experiment on mice has yielded data of the same order. Thus the administration of four times the LD<sub>50</sub> over a period of eight hours produced a total mortality of 27 per cent (Table III). The rate of elimination in milligrams per kilogram per hour appeared to be greater in mice than in rats.

TABLE III THE EFFECTS ON MICE OF MULTIPLE INTRAPERITONEAL DOSES OF 84-L RELATED AT SHORT INTERVALS

INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF MICE INJECTED	DOSE		MORTALITY	
		SINGLE (MG/KG)	CUMULATIVE (MG/KG)	PEP DOSE (%)	CUMULATIVE (%)
0	30	200	Initial dose	0	0
1	30	100	300	0	0
2	30	150	450	6.7	6.7
3	28	150	600	0	6.7
4	28	150	750	7.1	13.3
5	26	150	900	3.8	16.7
6	25	150	1050	8.0	23.3
7	23	150	1200	0	23.3
8	23	150	1350	4.3	26.7*
24	--	--	1350	59.1†	70.0†
72	--	--	1350	0	70.0

\*Twenty minutes after dose

†Since death from 84-L usually occurs within thirty minutes after the injection one hesitates to attribute this mortality to the pharmacologic action of the compound. However these figures do not affect the conclusions since 310 mg per kilogram in a single dose produced a mortality of 99 per cent.

Etherized dogs tolerated 60 to 70 mg per kilogram of 84-L given intravenously during a period of one hour. Dog 548 readily tolerated nine doses, each dose 5 mg per kilogram, during a period of ninety minutes (Fig 4) and Dogs 546 and 547 each received seventeen such doses in eighty minutes without endangering the respiration. Dog 549 (Fig 4) tolerated seven doses of 10 mg per kilogram during a period of sixty minutes but developed respiratory failure when the eighth dose was given on the seventieth minute. This dog was maintained without difficulty on artificial respiration. Dog 540 received four doses of 20 mg per kilogram during forty minutes. The fifth dose, which brought the total to 100 mg per kilogram in fifty-two minutes, produced respiratory failure. Artificial respiration maintained this animal in a satisfactory condition and twenty minutes after the fifth dose another injection of 20 mg per kilogram was made without producing circulatory failure.

Unanesthetized dogs have been given 100 mg per kilogram intraperitoneally twice daily for two days without producing signs of toxicity more severe than vomiting and mild muscular tremors (Table I). One hour after the injections the animals appeared normal.

#### *Chronic Toxicity—*

**Rats**—The intraperitoneal injection of 100 mg of 84-L per kilogram five days per week for fourteen weeks did not affect the rate of growth or produce any unfavorable reactions in male rats (Fig 2). The control and dosed groups each started with twenty animals. After fourteen weeks there were fifteen in the control and fourteen in the dosed group. At the end of the series of doses the mean hematologic findings on ten rats from each group were: (1) hemoglobin, grams per 100 cc—control, 13.1, dosed, 13.5, (2) red blood cells, millions per cubic millimeter—control, 8.3, dosed, 8.7, (3) white blood cells, thousands per cubic millimeter—control, 14.4, dosed, 19.3, (4) lymphocytes, per

cent control, 61, dosed, 79, (5) neutrophils, per cent control, 34, dosed, 19, (6) eosinophils, per cent control, 37, dosed, 10 The pathologist\* reported that no differences between the groups were found on examination of the tissues of the animals

**Rabbits**—Fifteen rabbits were given intraperitoneally, 50 mg per kilo gram of the drug for fourteen weeks The growth of the group was not significantly modified (Fig 2) During the period four animals died in the control

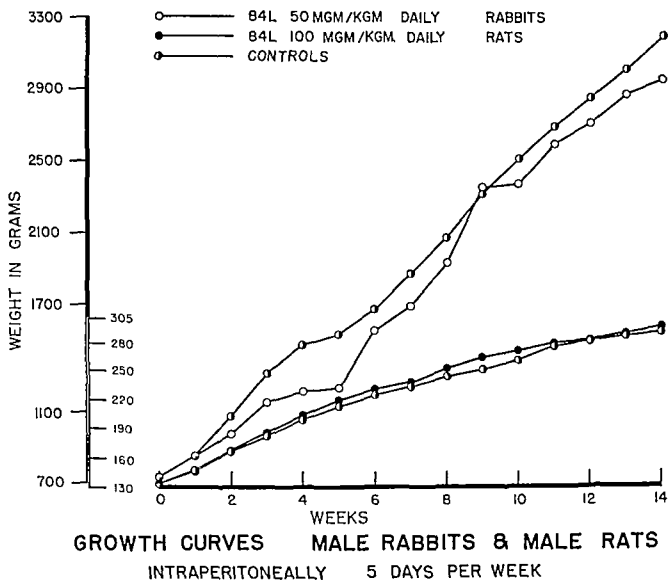


Fig 2

NUMBER OF ANIMALS	START	END
Control rabbits	1	11
Dosed rabbits	15	8
Control rats	20	15
Dosed rats	0	14

group and seven in the dosed but the distribution of deaths does not suggest that they were due to chronic effects of the drug After sixty six doses during ninety eight days the hematologic data revealed no difference between the control and the dosed groups The average results for the groups were (1) hemoglobin, grams per 100 cc control 111 dosed, 110, (2) red blood cells millions per cubic millimeter control 61 dosed 58 (3) white blood cells thousands per cubic millimeter control 86 dosed 103 (4) lymphocytes, per cent control, 69 dosed 70 (5) neutrophils per cent control 28 dosed 26

Dr F I Dessau Lederle Laboratories Division American Cyanamid Co made the examinations

*Dogs*—In a large series of dogs, Hewitt and associates<sup>1</sup> observed no evidence of chronic toxicity. Five of the dogs were given 50 mg per kilogram intraperitoneally twice a day for thirteen days and two were dosed orally with 25 mg per kilogram three times a day for sixty-four days. At the end of the period of dosing they were sacrificed and examined for pathologic changes,\* but none were found that could be attributed to the treatment given.

*Chicks*—Robbins' observation<sup>11</sup> that 2,4-dinitrophenol produced cataracts in the eyes of chicks prompted us to subject 84-L to a similar test. A group of eighteen 8-day-old White Rock chicks was divided into three balanced groups. Group 1 received a diet which contained 0.25 per cent 84-L and Group 2, 0.25 per cent 2,4-dinitrophenol, Group 3 was given the basal diet. Chicks on 2,4-dinitrophenol developed cataracts during the first twenty-four hours. The chicks in Group 1 were continued on the 84-L diet for fifteen days and there was never any evidence of lenticular changes. At the end of the experiment the lenses were sectioned and examined by a pathologist,† it was reported that the lenses were normal. The chicks fed 84-L grew at the same rate as the control animals in Group 3, and in appearance the individuals of the two groups were indistinguishable.

#### MISCELLANEOUS OBSERVATIONS —

*General Behavior* Intraperitoneal doses of 50 mg per kilogram given to dogs, cats, rats, and rabbits produced few signs. The dogs and rats were more sensitive to loud noises, but thirty minutes after the injections many of the animals in all the species tested appeared to be more quiet than usual, however, they were not asleep and responded normally to external stimuli.

Various tests disclosed that neither local anesthesia nor irritation were produced by 84-L.

*Isolated Intestine* The activity on isolated rabbit ileum is of a low order. Concentrations of 1/100,000 in Tyrode's solution were required to give a perceptible relaxation of normal or spastic strips. The drug produced no effect on the guinea pig intestine.

*Isolated Uterus* In concentrations of 1/100,000 84-L produced no effect on the isolated uterus from the rabbit or the rat. Virgin guinea pig uteri responded with weak contractions to a 1/100,000 concentration. In lower concentrations the response was barely perceptible or was absent.

*The antihistaminic action* on the guinea pig ileum was barely detectable and amounted to 1/2,000 to 1/10,000 of the activity of some of the clinically used compounds. Ten guinea pigs were injected intraperitoneally with 50 mg per kilogram of 84-L and thirty minutes later were subjected to a standardized spray of histamine. Nine animals convulsed in three minutes and of these one died. One guinea pig withstood the spray for ten minutes with no signs other than dyspnea. A retest of this guinea pig one and one-half hours after 84-L produced convulsions in seven minutes. All of a group of ten control guinea

\*See footnote \* page 221

†Dr. E. Woll, Lederle Laboratories Division, American Cyanamid Co.

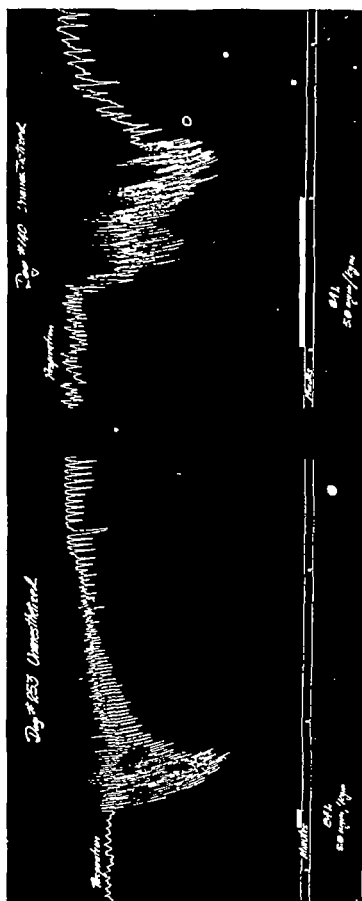


Fig. 3—Typical records of respiratory movements in unanesthetized dogs after intravenous injection of 84-J. The signal line marks the duration of the injection. The respiratory movements were relayed by a thread attached halfway between the umbilicus and the end of the sternum.

pigs convulsed in three minutes and three died. From these data we conclude that 84-L does not exaggerate the action of histamine but exerts little protection against it.

*Eye* Six cats were given the drug in a dosage of 25 mg per kilogram intraperitoneally and three were given 50 mg per kilogram. No evidence of myosis or mydriasis was observed. The local application of a 10 per cent solution produced no change in the pupil.

*Blood Sugar* In subconvulsant doses 84-L had no effect upon the blood sugar.<sup>12</sup>

*Diuretic Action* By the Lipschitz assay the diuretic potency is 17.5 times that of urea, or about one half the effectiveness of caffeine.

*Analgesia* In rats the compound produced evidence of a mild analgesia. The action was less than that produced by aminopyrine.

*Respiration* Changes in respiration have not been observed except after intravenous doses. In unanesthetized dogs the intravenous injection of 5 mg per kilogram in eight seconds stimulated the rate and depth of respiration for about a minute. Within two to three minutes from the start of the injection the respiration was normal (Fig. 3). When the same dose was injected over a period of sixty seconds, the stimulation was of a lower order and of a shorter duration. The injection of 20 mg per kilogram in eighteen seconds produced an intense stimulation of respiration which lasted about a minute. In three minutes the animal appeared to be normal. In etherized dogs the respiratory changes were much less prominent.

Intravenous doses of 2 to 15 mg per kilogram inhibited the respiration of normal and anesthetized rabbits. In unanesthetized rabbits the rapid injection of 5 mg per kilogram produced an apnea lasting from five to ten seconds. Recovery was complete in three minutes. A dose of 2 mg per kilogram injected in sixty seconds decreased the amplitude and slowed the respiration for thirty seconds. Twenty-five milligrams per kilogram injected in one minute killed one of two rabbits.

In anesthetized dogs lethal doses of 84-L produced death by respiratory failure. These animals could be maintained on artificial respiration long after breathing had ceased.

*Circulation* In etherized animals the response of the blood pressure to effective doses of 84-L (Fig. 4, Table IV) resembled that produced by epinephrine. The principal difference noticed was a longer duration of the depressor phase. Doses of 0.1 and 0.5 mg per kilogram were ineffective. Some dogs responded feebly to 1 mg per kilogram, but 5 mg per kilogram produced a quick, short-lasting rise in the blood pressure followed by a fall of somewhat longer duration. As the dose was increased the peak of the rise increased until it reached a maximum at about 20 mg per kilogram. The changes in the blood pressure produced by initial doses ranging from 5 to 40 mg per kilogram have been recorded in Table IV.

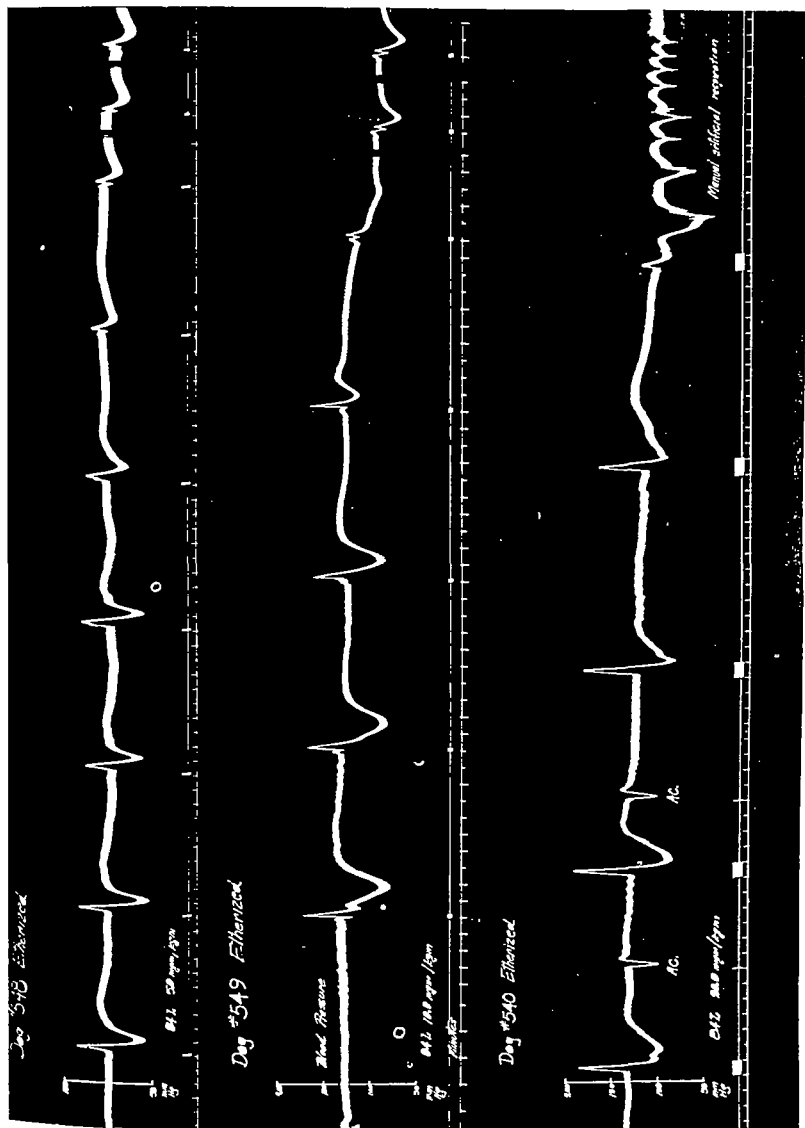


Fig. 4—The effect of repeated doses of 81 L on the blood pressure of etherized dogs. In each set the signal line marks the time for the repetition of the original dose.



TABLE IV THE EFFECT OF INITIAL INTRAVENOUS DOSES OF 84 L ON THE BLOOD PRESSURE OF ETHERIZED ANIMALS

ANIMAL	BLOOD PRESSURE CONTROL (MM HG)	INTRAVENOUS* DOSE (MG /KG )	BLOOD PRESSURE			
			RISE		FALL	
			MAXIMUM (MM HG)	DURATION (MIN )	MAXIMUM (MM HG)	DURATION (MIN )
Dog						
504	136	5	26	0.5	34	1.3
503	165	5	33	0.3	37	1.5
502	152	5	36	0.5	27	2.0
501	120	5	36	0.5	38	2.5
506	133	5	28	0.2	29	2.5
522	142	5	85	0.5	0	-
488	106	10	54	1.0	26	†
A 1	95	10	51	2.5	0	-
A 4	142	10	50	0.5	66	5.0
540	130	20	50	0.5	34	13 plus
A 2	120	20	92	1.2	40	9 plus
545	122	25	46	1.3	48	5 plus
489	112	25	28	1.0	52	†
490	115	25	39	2.0	48	†
491	145	25	91	4.0	0	-
544	156	25	38	1.0	50	5 plus
A 3	104	40	42	0.5	36	3.5
Cat						
498	136	10	12†	1.5	0	-
492	120	23	40	2.0	0	-
Rabbit						
495	98	10	42	15.0	0	-
493	68	25	26	3.0	0	-
494	88	25	Fatal dose			

\*The injection was completed in ten to thirty seconds

†Fall was interrupted by the injection of another compound

‡This rise was preceded by a fall of 8 mm Hg The duration of the fall was 0.3 minutes

The leg plethysmograph readily demonstrated that the sharp rise in blood pressure produced by intravenous doses of 84-L in dogs was accompanied by an equally sharp decrease in the volume of the leg (Fig 5). However, the vasodepressor phase is poorly reflected.

The close similarity between the response of the blood pressure to 84-L and to epinephrine, coupled with the observation that 84-L exaggerated the action of epinephrine, suggested that it inhibited either the cardiac vagus or those reactions which destroy epinephrine.

The vasodepressor response to stimulation of the right vagus before and after 84-L showed that the intravenous injection of 25 mg per kilogram blocked 25 to 100 per cent of the vagal activity for a period of thirty minutes (Table V). The exaggeration of the pressor response of epinephrine parallels the inhibition of the vagus. Doses of 5 and 10 mg per kilogram of 84-L produced essentially no vagal inhibition (Table V). The mechanism of inhibition is interesting since in doses which completely inhibited the cardiac vagus 84-L had no effect on the vasodepressor response to acetylcholine (Fig 6).

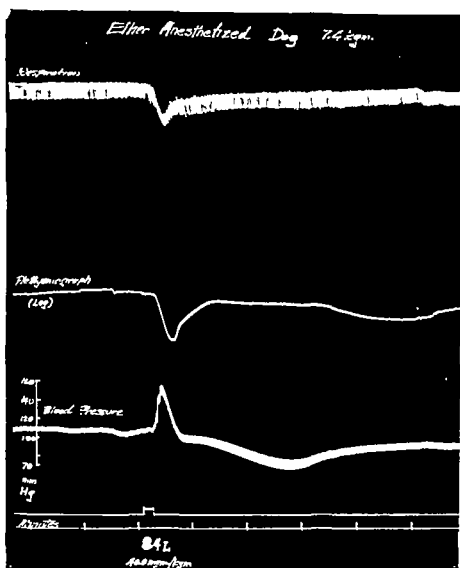


Fig 5—The effect of 84 L on the blood pressure leg volume and respiration of an etherized dog

TABLE V THE INHIBITION OF THE CARDIAC VAGUS BY 84 L

ANIMAL*	CONTROL VAGAL STIMUL FALL IN BLOOD PRESSURE (MM HG)	INTRAVENOUS DOSE (MG /KG )	MINUTES AFTER INJECTION		
			3	15	30
			VAGAL INHIBITION (%)		
Cat					
498	22	10 0	45	18	0
498	12	15 0	0		
498	16	25 0	100	81	81
492	31	25 0	80	61	0
492	23	25 0	56	39	0
Rabbit					
493	26	25 0	100		19
493	20	12 5	0		
Dog					
544	76	25 0	100	100	68
489	64	25 0	100	100	46
545	66	25 0	100	48	42
490	70	25 0	85	78	69
487	56	25 0	100	100	100†
488	60	10 0	0		
488	60	25 0	75‡		
491	100	25 0	50	60	25
542	74	5 0	8	0	
542	73	10 0	7		

The right vagus was used the blood pressure was recorded from the right carotid artery.  
 \*All animals except Dog 491 were anesthetized with ether. In Dog 491 the anesthetic was intravenous sodium pentobarbital.

†Complete inhibition for at least two hours.

‡Vagal activity was normal at forty five minutes

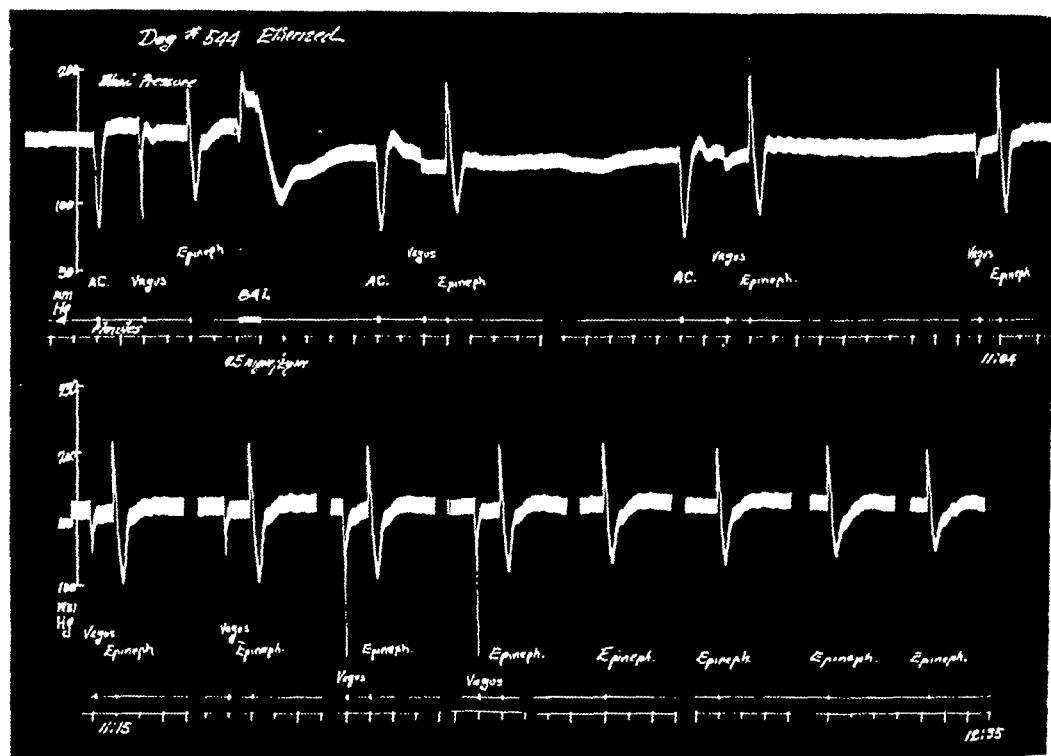


Fig 6—The action of 84-L on the response of the blood pressure to acetylcholine epinephrine and faradic stimulation of the right vagus. The doses per kilogram were 84-L 25 mg, acetylcholine 0.4  $\mu$ g and epinephrine hydrochloride 2 micrograms

Repeated injections produced a gradual diminution in the responses of the blood pressure and finally a complete disappearance of all action (Fig 4). These data may provide a key to the explanation of the disappearance of the headache which developed in some patients after a few doses<sup>2</sup>

Dogs with cords sectioned at approximately the second cervical vertebra and with all superior connections completely removed showed unmistakably that the vasopressor response of 84-L was not dependent upon centers in the brain. After this operation the vasopressor action of 84-L was exaggerated<sup>1</sup> (Fig 7). Rises of blood pressure of 100 to 180 mm Hg were common. Furthermore, the duration of the rise was much longer than in the etherized dogs. After the rise there was no fall unless the compound had been given before the blood pressure reached a stable level.

Electrocardiograms\* obtained from etherized dogs which had been injected intravenously with 5 to 50 mg of 84-L per kilogram revealed minimal changes. The heart rate was increased from 150 to 180 beats per minute during the hypertensive phase but returned to the initial rate during the hypotensive phase. Regular sinus rhythm was maintained and such changes in the form of the complex as were noted were those usually associated with rapid heart

\*The electrocardiographic studies were made by Dr Maynard B. Chenoweth, Cornell University Medical College.

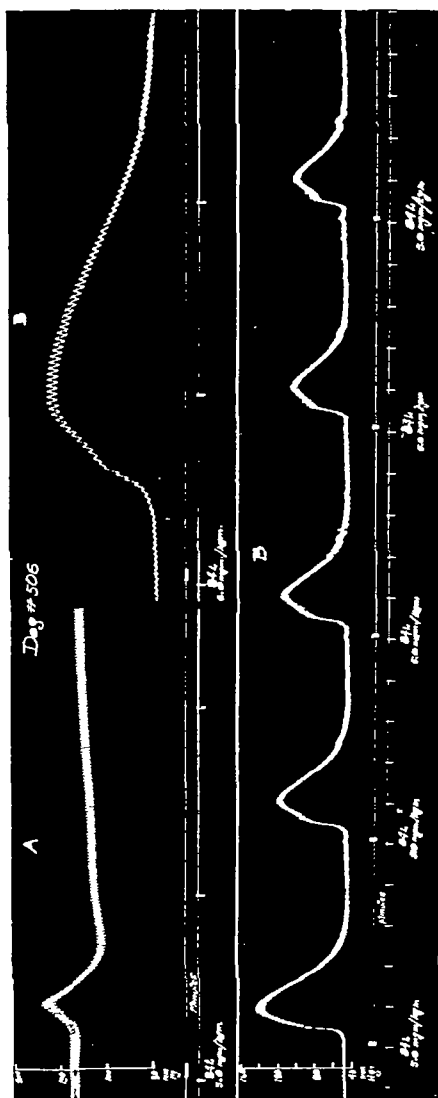


Fig —The effect of 84 I on the blood pressure before (I) and after (II) section of the cervical cord. Both v. and w. r. sectioned before the control injection (I). The injections in B were made at five minute intervals.

rates Electrocardiograms taken during the terminal stages revealed no changes not typical of anoxia

Cardiovascular responses in unanesthetized dogs In five unanesthetized dogs the rapid intravenous injection (ten seconds) of 5 mg of 84-L per kilogram produced an increase in blood pressure Expressed in millimeters of mercury the elevations were 68, 68, 68, 88, and 118\* When the same dose was given in sixty seconds the rise was 31 mm Hg Only two dogs were tested These data indicated that the cardiovascular system of an unanesthetized dog was more sensitive to 84-L than that of the etherized animal Electrocardiograms reflected similar differences Although the rapid intravenous injection of 5 mg per kilogram of 84-L produced insignificant changes in the electrocardiogram of etherized dogs, the same dose in unanesthetized dogs produced a number of changes in the rate and pacemaker location Sinus arrest for periods as long as nine seconds and irregular rapid sinus rhythm interspersed with premature junctional and ventricular contractions were observed Approximately five minutes after the injection the electrocardiogram was normal Doses of 0.1 to 0.5 mg per kilogram produced no changes or only slight changes in the rate and form of the electrocardiogram

#### FATE OF COMPOUND —

The absence of accumulative effects after multiple doses of 84-L (Tables II and III) suggested that the animals quickly became highly tolerant or that the compound was rapidly destroyed or excreted Although a certain vascular tolerance to the drug developed, no comparable tolerance was seen in any other system

*Liver* —The role of the liver in the detoxication was examined by subjecting rats first to a series of doses of carbon tetrachloride and then, before hepatic recovery, to multiple doses of 84-L The quantity of 84-L used slightly exceeded that which the rat was able to destroy or excrete so that any limitation in the ability of a key organ to perform its function would be reflected in a greater incidence of reactions in the test group Rats weighing from 180 to 210 grams were given orally 0.166 cc of carbon tetrachloride per 100 grams of body weight This dose was diluted to 0.5 cc with corn oil (Mazola) and given on days 1, 2, 3, 4, 5, 7, and 8 The tests for liver damage and 84-L catabolism were made on the first day after the last dose of carbon tetrachloride The function of the liver was tested by following the duration of the anesthesia produced by sodium pentobarbital, a drug which is destroyed principally in the liver<sup>13</sup> The results shown in Table VI demonstrate that a dose of 30 mg per kilogram of sodium pentobarbital anesthetized 95 to 100 per cent of the control and the carbon tetrachloride-treated rats Duration of the anesthesia as measured by the righting reflex was one hour in the control group and seven hours in the treated group (Table VI) The administration of 84-L in multiple graduated doses to rats treated similarly with carbon tetrachloride gave little evidence of an exaggeration of the effects seen in normal rats (Table VII) These data suggest that the liver is unimportant in the detoxication of 84-L

\*Coagulation of blood interfered with the evaluation of the depressor phase

TABLE VI THE EFFECT OF PREPOSING WITH CARBON TETRACHLORIDE ON THE DURATION OF ANESTHESIA PRODUCED BY SODIUM PENTOBARBITAL IN RATS A TEST FOR LIVER FUNCTION

TIME AFTER SODIUM PENTOBARBITAL* (HOURS)	PERCENTAGE OF RATS WITHOUT RIGHTING REFLEX	
	TREATED† (7 DOSES CCL ‡)	CONTROL†
0.5	95	100
0.7	95	70
1.0	95	5
2.0	95	0
3.0	95	0
4.0	100	0
5.0	100	0
6.0	100	0
7.0	94	0
7.0+	86	0
Overnight	23	0

\*Thirty milligrams of sodium pentobarbital per kilogram intraperitoneally

†Twenty rats weight 140 to 170 grams at time of test.

‡The carbon tetrachloride was dissolved in corn oil (Mazola) 1 c.c. diluted to 3 c.c. the dose 0.5 c.c. of the mixture per 100 grams of body weight was given orally on days 1 2 3 4 5 7 and 8

TABLE VII THE MORTALITY PRODUCED BY 84 L GIVEN IN MULTIPLE DOSES TO RATS PRETREATED WITH CARBON TETRACHLORIDE\*

GROUP†	INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF RATS INJECTED	DOSE		MORTALITY	
			SINGLE (MG/KG)	CUMULATIVE (MG/KG)	PER DOSE (%)	CUMULATIVE (%)
Treated (7 doses of CCl <sub>4</sub> )	0	29	300	Initial dose	10	10
	1	--	--	--	--	--
	2	26	100	400	0	10
	3	26	100	500	0	10
	4	26	100	600	4	14
	5	25	100	700	4	17
	6	24	100	800	0	17
	7	24	100	900	12	27
Control	0	20	300	Initial dose	5	5
	1	--	--	--	--	--
	2	19	100	400	0	5
	3	19	100	500	0	5
	4	19	100	600	0	5
	5	19	100	700	0	5
	6	19	100	800	0	5
	7	19	100	900	10	15

The dose of carbon tetrachloride is given in footnote ‡ to Table VI

†Twenty treated rats and twenty control rats weight range in grams 140 to 180

*Kidneys*—The administration of multiple doses of 84 L to unilaterally and bilaterally nephrectomized rats demonstrated the importance of the kidney in the elimination of this compound (Tables II and VIII). In sixty rats the left kidney was removed and after four or five days the animals were injected intraperitoneally with repeated doses of 84 L. The schedule of doses was 300 mg per kilogram initially followed on hours 2, 3, 4, 5, 6, 7, and 8 by additional doses of 100 mg per kilogram. Thus, in a period of eight hours the total cumulative dose was 1,000 mg per kilogram. With this schedule the accumulated mortality for the eight hours was 80 per cent in the unilaterally nephrectomized rats and 34 per cent in the normal rats.

rates. Electrocardiograms taken during the terminal stages revealed no changes not typical of anoxia.

Cardiovascular responses in unanesthetized dogs. In five unanesthetized dogs the rapid intravenous injection (ten seconds) of 5 mg of 84-L per kilogram produced an increase in blood pressure. Expressed in millimeters of mercury the elevations were 68, 68, 68, 88, and 118\*. When the same dose was given in sixty seconds the rise was 31 mm Hg. Only two dogs were tested. These data indicated that the cardiovascular system of an unanesthetized dog was more sensitive to 84-L than that of the etherized animal. Electrocardiograms reflected similar differences. Although the rapid intravenous injection of 5 mg per kilogram of 84-L produced insignificant changes in the electrocardiogram of etherized dogs, the same dose in unanesthetized dogs produced a number of changes in the rate and pacemaker location. Sinus arrest for periods as long as nine seconds and irregular rapid sinus rhythm interspersed with premature junctional and ventricular contractions were observed. Approximately five minutes after the injection the electrocardiogram was normal. Doses of 0.1 to 0.5 mg per kilogram produced no changes or only slight changes in the rate and form of the electrocardiogram.

#### FATE OF COMPOUND —

The absence of accumulative effects after multiple doses of 84-L (Tables II and III) suggested that the animals quickly became highly tolerant or that the compound was rapidly destroyed or excreted. Although a certain vascular tolerance to the drug developed, no comparable tolerance was seen in any other system.

*Liver* — The role of the liver in the detoxication was examined by subjecting rats first to a series of doses of carbon tetrachloride and then, before hepatic recovery, to multiple doses of 84-L. The quantity of 84-L used slightly exceeded that which the rat was able to destroy or excrete so that any limitation in the ability of a key organ to perform its function would be reflected in a greater incidence of reactions in the test group. Rats weighing from 180 to 210 grams were given orally 0.166 cc of carbon tetrachloride per 100 grams of body weight. This dose was diluted to 0.5 cc with corn oil (Mazola) and given on days 1, 2, 3, 4, 5, 7, and 8. The tests for liver damage and 84-L catabolism were made on the first day after the last dose of carbon tetrachloride. The function of the liver was tested by following the duration of the anesthesia produced by sodium pentobarbital, a drug which is destroyed principally in the liver<sup>13</sup>. The results shown in Table VI demonstrate that a dose of 30 mg per kilogram of sodium pentobarbital anesthetized 95 to 100 per cent of the control and the carbon tetrachloride-treated rats. Duration of the anesthesia as measured by the righting reflex was one hour in the control group and seven hours in the treated group (Table VI). The administration of 84-L in multiple graduated doses to rats treated similarly with carbon tetrachloride gave little evidence of an exaggeration of the effects seen in normal rats (Table VII). These data suggest that the liver is unimportant in the detoxication of 84-L.

\*Coagulation of blood interfered with the evaluation of the depressor phase.

TABLE VI THE EFFECT OF PREDOSING WITH CARBON TETRACHLORIDE ON THE DURATION OF ANESTHESIA PRODUCED BY SODIUM PENTOBARBITAL IN RATS, A TEST FOR LIVER FUNCTION

TIME AFTER SODIUM PENTOBARBITAL (HOURS)	PERCENTAGE OF RATS WITHOUT RIGHTING REFLEX	
	TREATED† (7 DOSES CCL ‡)	CONTROL†
0.5	95	100
0.7	95	70
1.0	95	5
2.0	95	0
4.0	95	0
5.0	100	0
6.0	100	0
7.0	94	0
7.0+	86	0
Overnight	23	0

Thirty milligrams of sodium pentobarbital per kilogram intraperitoneally

†Twenty rats weight 140 to 170 grams at time of test.

‡The carbon tetrachloride was dissolved in corn oil (Mazola) 1 cc diluted to 3 cc the dose 0.5 cc. of the mixture per 100 grams of body weight was given orally on days 1, 3, 4, 5, and 8

TABLE VII THE MORTALITY PRODUCED BY 84 L GIVEN IN MULTIPLE DOSES TO RATS PRETREATED WITH CARBON TETRACHLORIDE\*

GROUP†	INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF RATS INJECTED	DOSE		MORTALITY	
			SINGLE (MG/KG)	CUMULATIVE (MG/KG)	PER DOSE (%)	CUMULATIVE (%)
Treated (7 doses of CCL)	0	29	300	Initial dose	10	10
	1	—	—	—	—	—
	2	26	100	400	0	10
	3	26	100	500	0	10
	4	26	100	600	4	14
	5	25	100	700	4	17
	6	24	100	800	0	17
	7	24	100	900	12	27
Control	0	20	300	Initial dose	5	5
	1	—	—	—	—	—
	2	19	100	400	0	5
	3	19	100	500	0	5
	4	19	100	600	0	5
	5	19	100	700	0	5
	6	19	100	800	0	5
	7	19	100	900	10	15

The dose of carbon tetrachloride is given in footnote ‡ to Table VI.

†Twenty treated rats and twenty control rats weight range in grams 140 to 170

**Kidneys**—The administration of multiple doses of 84 L to unilaterally and bilaterally nephrectomized rats demonstrated the importance of the kidney in the elimination of this compound (Tables II and VIII). In sixty rats the left kidney was removed and after four or five days the animals were injected intraperitoneally with repeated doses of 84 L. The schedule of doses was 300 mg per kilogram initially followed on hours 2, 3, 4, 5, 6, 7, and 8 by additional doses of 100 mg per kilogram. Thus, in a period of eight hours the total cumulative dose was 1,000 mg per kilogram. With this schedule the accumulated mortality for the eight hours was 80 per cent in the unilaterally nephrectomized rats and 34 per cent in the normal rats.



There were two groups of bilaterally nephrectomized rats (Table VIII). In one group the total nephrectomy was accomplished six days after the removal of the left kidney. The 84-L was administered twenty-four hours after the last operation. The initial dose was 200 mg per kilogram with subsequent doses of 100 mg per kilogram given on the second, third, fourth, and fifth hours. After an accumulated dose of 400 mg per kilogram 50 per cent of the rats were dead, and after 600 mg per kilogram the mortality was 100 per cent.

TABLE VIII THE MORTALITY PRODUCED BY 84 L GIVEN IN MULTIPLE DOSES TO NEPHRECTOMIZED RATS

GROUP†	INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF RATS INJECTED	DOSE		MORTALITY	
			SINGLE (MG/KG)	CUMULATIVE (MG/KG)	PEP DOSE (%)	CUMULATIVE (%)
I	0	60	300	Initial dose	58	58
Unilateral	1	--	---	---		
nephrectomy	2	25	100	400	0	58
45 days be	3	25	100	500	0	58
fore initial	4	25	100	600	16	65
dose	5	21	100	700	5	67
	6	20	100	800	15	72
	7	17	100	900	24	78
	8	13	100	1000	8	80
II	0	28	200	Initial dose	18	18
Left kidney	1	--	---	---		
removed 6	2	23	100	300	17	33
days before	3	19	100	400	26	50
initial dose,	4	14	100	500	71	86
right kidney	5	4	100	600	100	100
removed 24						
hours before						
initial dose						
III	0	15	200	Initial dose	0	0
Total neph	1	--	---	---		
rectomy	2	15	100	300	0	0
½ 2 hours	3	15	100	400	7	7
before ini	4	14	100	500	44	47
tial dose	5	8	100	600	75	87

\*Effect of 84 L on normal rats is summarized in Table II Group I

†Weight range in grams Groups I and II 170 to 200 Group III 204 to 240

In normal rats the LD<sub>50</sub> for a single dose was 465 mg per kilogram (Fig 1) and a single dose of 600 mg per kilogram produced a mortality of 75 per cent. When the same schedule of doses was used on a group of rats which had been bilaterally nephrectomized under ether one-half to two hours before the first dose of 84-L, an accumulated dose of 500 mg per kilogram killed eight of fifteen rats and 600 mg per kilogram produced a mortality of 87 per cent. As an additional control on mortality attributable to the operation, the survival of thirteen totally nephrectomized rats was followed. Twenty-four hours after the operation the survival was 85 per cent and after forty eight hours, 54 per cent. Attention should be called to the fact that the residual effects of ether in rats which were dosed on the same day that they were operated provided a significant protection against the lower doses of 84-L.

The value of the kidney in the elimination of 84 L has been shown graphically in Fig 8. Here the mortality in per cent has been plotted on logarithmic probability paper for the cumulative doses administered to normal and to unilaterally and bilaterally nephrectomized rats, and these curves have been compared with the standard intraperitoneal dosage mortality curve (Fig 1). The mortality for the cumulative doses in the normal rats did not change significantly after the first dose. In the unilaterally nephrectomized rats the mortality from the initial dose was higher than in the unoperated animals and con

#### EFFECT OF NEPHRECTOMY ON INTRAPERITONEAL TOXICITY OF 84 L IN RATS

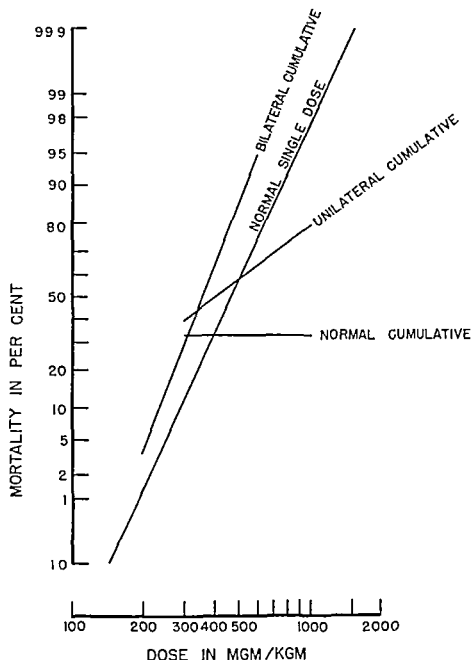


Fig 8—The schedule for the cumulative dosing of normal and unilaterally nephrectomized rats was: initial dose 300 mg per kilogram at zero time and 100 mg per kilogram at 3, 4, 6, 7 and 8 hours. For the bilaterally nephrectomized rats the schedule was: initial dose 200 mg per kilogram at zero time and 100 mg per kilogram at 3, 4 and 5 hours.

tinued to mount as the doses accumulated. However, the mortality from these accumulated doses did not equal that produced by equivalent single doses. The dosage mortality curve for the accumulated doses in the bilaterally nephrectomized rats paralleled and closely approximated that for the single doses to normal rats.

*Excretion*—Although the data indicate that the elimination of the compound is accomplished principally by the kidney, the form in which it is excreted has not been determined. No practical chemical test for small quantities of the compound has been devised. However, a bio assay on filaria-infested rats indicated that the equivalent of 63 per cent of a 300 mg per kilogram intraperitoneal dose was excreted during the first twenty-three hours. In spite of the fact that nothing is known about the metabolic fate of 84-L, the low antifilarial activity of its relatives<sup>14</sup> strongly suggests that, in the rat, most of the compound is excreted unchanged.

#### SUMMARY AND CONCLUSIONS

1-Diethylcarbamy-4-methylpiperazine hydrochloride, also known as Hetiazan and 84-L, has a low toxicity and causes few side reactions.

The intraperitoneal LD<sub>50</sub> in mice was 248 mg per kilogram and in rats, 465 mg per kilogram. The oral LD<sub>50</sub> in mice was 660 mg per kilogram and in rats, 1,380 mg per kilogram.

Mice, rats, rabbits, and dogs readily tolerated intraperitoneal injections of 100 mg per kilogram.

Daily intraperitoneal doses of 50 mg per kilogram in rabbits and 100 mg per kilogram in rats, five days per week for fourteen weeks, produced no evidence of toxicity. Twenty-five milligrams per kilogram twice a day, orally, for two months produced no evidence of toxicity in dogs.

The compound was not irritating, it produced no local anesthesia and had no effect on the eye, on the isolated uterus or intestine, or on the blood sugar. It was mildly diuretic and analgesic. Intravenous doses of 2 to 25 mg per kilogram in unanesthetized dogs stimulated the respiration. The heart and blood pressure were not affected by rapid intravenous injections of 0.5 mg per kilogram, but larger intravenous doses in unanesthetized dogs produced a transient deviation from the normal.

The compound was rapidly excreted by the kidney. In rats and mice the rate per hour was approximately one-third of the intraperitoneal LD<sub>50</sub>.

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# LABORATORY METHODS

## SCREENING METHOD FOR BLOOD GLUCOSE\*

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IN THE course of mass surveys carried out by this Section<sup>1</sup> it became increasingly evident that a considerable saving of time and money might be effected through elimination of definitely normal blood samples from routine glucose analysis by means of a simplified test. The method is based on that of Hagedorn and co-workers<sup>2</sup> but the steps requiring experience in chemical techniques and fluid reagents have been eliminated. The time required for a single analysis is about five minutes, the amount of blood, 0.1 milliliter. This paper describes the screening method and gives the results obtained with the first fifty blood samples.

### REAGENTS AND APPARATUS

All reagents are in tablet form. In Boston (and presumably in many other localities) ordinary tap water may be used instead of distilled water.

#### Tablet 1

ZnSO <sub>4</sub> 7H <sub>2</sub> O	10 mg
NaCl	190 mg
Talc	As binder
Mineral oil	As lubricant

#### Tablet 2

KI	100 mg
NaHCO <sub>3</sub>	10 mg

#### Tablet 3

K <sub>3</sub> [Fe(CN) <sub>6</sub> ], recrystallized	1.28 mg
Na <sub>2</sub> CO <sub>3</sub> , anhydrous	65 mg
Starch, soluble	1 mg
NaCl	92 mg

#### Tablet 4

Tartaric acid	80 mg
ZnSO <sub>4</sub> 7H <sub>2</sub> O	20 mg
Starch	As binder

#### Heating Tablets

Methenamine ||

The apparatus consists of a Pyrex test tube 16 by 150 mm, graduated at 5 ml, the mouth of which is widened into a funnel, a scoop made of a piece of glass rod, and a portable test tube stand. The stand is so constructed that the

\*United States patent applied for

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From the United States Public Health Service Diabetes Section

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§We gratefully acknowledge the generous help of the staff of Eli Lilly & Company Indianapolis Ind who prepared and furnished reagent tablets

||Reagent tablets No 1588 Eli Lilly & Company Indianapolis Ind

test tube can be heated in a reproducible manner by the heating tablets, that is the distance between tube and tablets is constant (five eighths of an inch) and drafts are kept out

#### PROCEDURE

Fill the test tube with water to the 5 ml mark. Obtain 0.1 ml capillary blood from the fingertip or ear lobe by means of a capillary pipette. Deliver and rinse the blood into the water in the tube. Add one Tablet 1 and one Tablet 2 to the tube, put it on the stand, and light two heating tablets underneath it. The blood proteins are coagulated by the zinc hydroxide and form a scum which floats on top of the liquid. As the solution boils, the cake of protein is pushed upward in the test tube by the steam and can be removed with the glass scoop when it reaches the funnel shaped mouth. The funnel prevents the solution from boiling over and unremoved protein from falling back into the tube. When both heating tablets are nearly consumed, light another heating tablet on them and add one Tablet 3 to the test tube. At the end of the second heating period cool the test tube by immersion in cold water and add Tablet 4. When cold, the solution will be either blue, due to the formation of the starch iodine complex, or colorless, if no iodine is present.

#### RESULTS

The results of the screening method were checked against the blood sugar method of Somogyi<sup>3, 4</sup> as modified by Nelson<sup>5</sup>. In order to obtain a more accurate value for the true sugar concentration of the blood, the Somogyi Nelson determination was carried out on a 10 ml sample of venous blood, the 0.1 ml sample for the screening method being taken from the same sample of venous blood. The samples were taken from normal and diabetic subjects.\*

Fifty blood samples were subjected to the screening method, then analyzed by the Somogyi Nelson method. Twenty five samples having a true sugar concentration of 170 mg per cent or less by the Somogyi Nelson method, gave a blue color (negative reaction) in the screening method. Four samples had blood sugar values between 170 and 180 mg per cent and gave faint or dark blue colors (negative) or no color (positive) reaction. None of the twenty one blood samples containing more than 180 mg per cent gave a color reaction in the screening test.

#### DISCUSSION

The amount of potassium ferricyanide in Tablet 3 is so adjusted that it will be completely reduced under the conditions of the test by the glucose present in 0.1 ml blood when the concentration is 180 mg per cent or more. If the glucose concentration in the blood sample is lower than 170 mg per cent, some potassium ferricyanide is left over to oxidize the potassium iodide in Tablet 1 to iodine which produces a blue color with starch upon acidification by Tablet 4. The variability of the results between 170 and 180 mg per cent is due to variation in the tablets, the amount of water, heating, and so forth.

\*We wish to thank Dr. A. Marble of the New England Deaconess Hospital for making available the clinical material.

The 180 mg per cent level for capillary glucose has been chosen arbitrarily as the value above which a retest by a conventional blood sugar method seems warranted. Naturally, this level may be adjusted at will by altering the conditions of the test, for example the potassium ferricyanide content of Tablet 3. Inasmuch as a knowledge of the actual blood sugar value is less important in screening diagnostic work than whether or not a certain level is exceeded, the test recommends itself wherever laboratory facilities are unavailable.

#### SUMMARY

A simple and inexpensive screening method for blood glucose has been described. A blood sample of 0.1 ml. may be classified as above or below a certain glucose concentration within five minutes by means of this test. In the first fifty cases examined by this method, twenty-five blood samples below 170 mg per cent glucose gave a negative result, twenty-one samples above 180 mg per cent a positive result, and four samples between 170 and 180 mg per cent both positive and negative results.

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## AN INCREASE OF COMPLEMENT UNITS BY THE USE OF EGG ALBUMIN

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THE behavior of complement and amboceptor titrations in complement fixation tests for the serodiagnosis of syphilis has been under study in Santa Rosa Hospital Laboratory for some time. Literature pertaining to the subject is meager.

Periodically difficulty has been encountered, particularly in the complement titration, that was unexplainable from the standpoint of the pH or the reagents used.

Amboceptor titrations resulted in a unit well within the usable range, but the complement titration was unsatisfactory for use, with complete hemolysis in perhaps only the first one or two tubes.

All guinea pigs used for complement had been tested previously for titers and complement fixation and found to be satisfactory. Blood from five guinea pigs was pooled. Since lyophilized complement titrations occasionally exhibited similar reactions, complement per se was eliminated as a possible cause of the difficulty. Inhibition of hemolysis was noted in the complement titrations containing antigen. The pH of the saline was always within usable range.

The absence of albumin in the titration was considered as a possible cause of the difficulty encountered. Varying amounts of pooled negative serum were found to be unsatisfactory as a source of albumin, but the addition to each tube in the titration of 0.2 cc of 50 per cent egg albumin in 0.85 per cent saline as previously recommended by Kolmer for testing spinal fluids, was found satisfactory.

Parallel titrations using the same reagents, except for the absence of the 0.2 cc of 50 per cent egg albumin in 0.85 per cent saline, resulted in the titrations given in Table I.

Supplementary titrations following the eighteen-hour incubation of the complement fixation tests rarely result in a change of amboceptor dilution when the units for the test have been determined by a preliminary titration using the 0.2 cc amounts of 50 per cent egg albumin in each tube of the titration. Whereas, the supplementary titrations after the incubation period on tests in which the unit of complement has been determined in preliminary titrations not using the egg albumin, frequently necessitate an increased dilution of amboceptor for tests with a maximum sensitivity.

Variations in the unit of complement with cardiolipin lecithin antigen and regular antigen have been reported previously<sup>1</sup> and the variation is consistent whether or not egg albumin is used.

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TABLE I

WITH EGG		WITHOUT EGG	
AMBOCEPTOR 1 UNIT	COMPLEMENT	AMBOCEPTOR 1 UNIT	COMPLEMENT
10,000	1 37	10,000	1 30
12,000	1 43	12,000	1 30
12,000	1 33	12,000	Only 5 tube hemolyzed
12,000	1 43	12,000	1 30
12,000	1 37	12,000	1 33
12,000	1 43	12,000	1 33
12,000	1 43 Kolmer	12,000	1 37
12,000	1 50 Cardio Kolmer		
16,000	1 33	16,000	1 30
16,000	1 43	16,000	1 30
16,000	1 43	10,000	1 33
16,000	1 33	12,000	1 33
16,000	1 43 Kolmer	12,000	1 33
	1 50 Cardio Kolmer		
16,000	1 37	16,000	1 33
16,000	1 43	16,000	1 33
16,000	1 43 Kolmer	16,000	1 33
	1 50 Cardio Kolmer		
16,000	1 50	16,000	1 33
16,000	1 37	16,000	1 37
16,000	1 43	16,000	1 37
16,000	1 50	16,000	1 37
20,000	1 37	20,000	1 30
20,000	1 43	20,000	1 30
20,000	1 33 Kolmer	20,000	1 33 Kolmer
	1 37 Cardio Kolmer		1 33 Cardio Kolmer
20,000	1 43 Kolmer	10,000	1 37
	1 50 Cardio Kolmer		
20,000	1 37 Kolmer	20,000	1 37
	1 43 Cardio Kolmer		

## CONCLUSION

The use of 0.2 cc of 50 per cent egg albumin in normal saline in each tube of the preliminary amboceptor and complement Kolmer titrations usually results in a higher unit of complement and frequently in an increased dilution of amboceptor to be used in the Kolmer complement fixation test for syphilis. With the addition of the 50 per cent egg albumin, supplementary titrations following the incubation period rarely result in the necessity for a change in the amboceptor dilution to obtain maximum sensitivity.

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# A COLORIMETRIC METHOD FOR THE DETERMINATION OF MICROQUANTITIES OF ETHANOL IN BLOOD AND OTHER BIOLOGIC FLUIDS

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WITH THE TECHNICAL ASSISTANCE OF RENE F HIRSCH

THE principal methods for the determination of microquantities of ethanol involve its oxidation followed by measurement of the resultant equivalent reduction. Various oxidizing agents have been used<sup>1, 3</sup> but the majority of methods are based on the work of Widmark<sup>4</sup> and Nicloux and co workers<sup>5</sup> and use an acid solution of potassium dichromate. Investigators have varied the procedure of distilling the alcohol into the acid dichromate and both titrimetric<sup>6, 8</sup> and colorimetric<sup>9, 11</sup> methods have been used to determine the reduced dichromate. The maximal sensitivity of these methods is approximately 20  $\mu$ g of ethanol. These methods are not specific, since any volatile substance oxidizable by dichromate will be determined as ethanol. The presence of such substances as methanol, formaldehyde, acetone, and other aldehydes and ketones gives high results.<sup>10, 11, 13</sup> The accuracy of these methods in the determination of ethanol is open to question unless such substances can be removed or are known not to be present in quantities causing interference. This is especially important in the determination of the ethanol level in blood for medicolegal purposes.

A new method for the determination of ethanol has been devised involving the conversion of ethanol to acetaldehyde and the determination of the latter colorimetrically with phydroxydiphenyl.<sup>14, 15</sup> This method is considerably more sensitive and more specific than pre existing methods. Furthermore it retains the advantages of requiring no special apparatus and of being simple to carry out.

## PROCEDURE

Up to 20 cc of the sample to be analyzed are placed with several glass beads in a 50 cc single side arm distillation flask connected with a small Liebig condenser. Distilled water is added to 20 cc, followed by 0.1 cc 10 per cent NaOH. The flask is closed tightly with a rubber stopper and at least 5 cc distilled. The entire distillate is then washed into the flask of an identical setup the flask in this case containing 25 cc saturated aqueous K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> (CP), 0.5 cc concentrated H<sub>2</sub>SO<sub>4</sub> (CP), and several glass beads (Care must be taken to prevent any of the dichromate from entering the side arm since dichromate interferes with the color reaction). The flask is stoppered immediately with a rubber stopper and the acetaldehyde formed is distilled into a graduated receptacle such as a centrifuge tube, in an ice bath.\* The volume that must be distilled to obtain all the

From Camp Detrick

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What percentage of the ethanol was oxidized to acetic acid was not determined. Consistency of the results obtained indicates that the fraction of the ethanol completely oxidized remained constant under the conditions of the procedure outlined. Distillation from the dichromate-sulfuric acid mixture was begun immediately after addition of the ethanol and no differences in results were observed whether this mixture was hot or cold at the time of addition of the ethanol. As soon as one sample has been distilled another can be added to the reaction mixture. Thus several samples can be distilled in succession without changing the dichromate-sulfuric acid mixture.

acetaldehyde formed varies directly with the amount of ethanol present in the original sample. It was found that if the original sample contains 20  $\mu\text{g}$  of ethanol at least 5 c c must be distilled, for 10  $\mu\text{g}$  of ethanol a distillation of 2 c c suffices.

The acetaldehyde in the distillate is then determined colorimetrically by reaction with p hydroxydiphenyl<sup>14, 15</sup>. One cubic centimeter of the distillate is placed in an 18 by 150 mm Pyrex test tube. One drop of 5 per cent  $\text{CuSO}_4$  is added, followed by 6.00 c c of concentrated  $\text{H}_2\text{SO}_4$  (special, As and N free) added slowly, with shaking, in an ice bath. One tenth cubic centimeter of 1.5 per cent p hydroxydiphenyl in 0.5 per cent NaOH is added directly to the solution and dispersed by vigorous shaking. The tube is placed in a 30° C water bath and after approximately fifteen minutes the reagent is redispersed by shaking. After incubation for at least thirty minutes the tube is placed in a boiling water bath for ninety seconds to dissolve excess reagent. The tube is then placed immediately in an ice bath and brought to room temperature. The color (deep violet) is read in a photoelectric colorimeter against a sulfuric acid reagent blank which has been treated as described (except that 1.00 c c of water is used instead of the distillate). A filter having peak transmittance at approximately 560  $\text{m}\mu$  is used. The color can be read immediately and is stable for several hours.

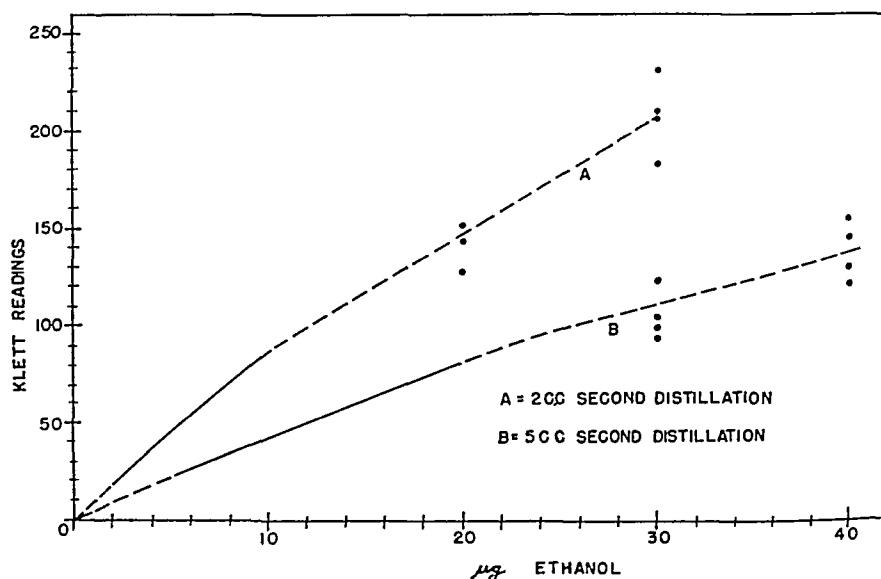


Fig 1—Color intensity obtained with known quantities of ethanol

In our experiments the Klett Summerson photoelectric colorimeter was used with a Klett No 54 filter. Fig 1 presents graphically the results of distilling 2 and 5 c c quantities in the second distillations, using known concentrations of ethanol. The solid portions of the curves were drawn from the experimental points (20 for curve A, 32 for curve B) by the method of least squares. It is apparent from the graph that the maximal amounts of ethanol falling on the straight line (solid) portions of the curves for the 2 and 5 c c distillations are approximately 10 and 20  $\mu\text{g}$ , respectively. Several experimental points above these limits are plotted in Fig 1 to show the variability of results obtained at higher concentrations.

**Sensitivity and Precision**—The sensitivities for the 5 and 2 c c distillations are approximately 1 and 0.5  $\mu\text{g}$  of ethanol, respectively. It was found that the sensitivity could be increased to approximately 0.2  $\mu\text{g}$  by distilling only 1.20 c c in the second distillation. Thus, if a 10 c c sample is available a concn

tration of 0.02  $\mu\text{g}$  per cubic centimeter can be determined. The standard error of a single determination in the range between 5 and 20  $\mu\text{g}$  of ethanol for the 5 c.c. distillation is 5 per cent and between 2 and 10  $\mu\text{g}$  of ethanol for the 2 c.c. distillation, 7 per cent. Since nineteen times out of twenty a single determination will be within  $\pm 2$  standard errors of the true value, the error of a single determination is  $\pm 10$  per cent for the 5 c.c. distillation and  $\pm 14$  per cent for the 2 c.c. distillation\* (The experimental error is greater at or near the limit of sensitivity).

The error, of course, can be reduced by running replicates. The number of replicates required (N) to give a desired precision (P) can be computed by substitution in the following formulas

$$N = \frac{100}{(P)} \quad (\text{for 5 c.c. second distillation})$$

$$N = \frac{196}{(P)} \quad (\text{for 2 c.c. second distillation})$$

*Interfering Substances*—The following substances did not give color in 100  $\mu\text{g}$  amounts, distilling 2 c.c. in the second distillation: dihydroxyacetone, fructose, D xylose, D ribose, succinic acid, fumaric acid, citric acid, malic acid, cis aconitic acid,  $\alpha$  ketoglutaric acid, acetone, ascorbic acid, tartaric acid, urea, glycine, tryptophane, methionine, methanol, glucose, pyruvic acid, glycerol, acetic acid, and lactic acid (up to 500  $\mu\text{g}$ ).

In Table I are listed those substances which were found to give color, distilling 2 c.c. on the second distillation. One hundred microgram amounts were used and the interference is given in terms of micrograms of ethanol giving an equivalent intensity of color. Obviously, acetaldehyde itself would give the color reaction.

TABLE I SUBSTANCES INTERFERING IN THE DETERMINATION OF ETHANOL

INTERFERING SUBSTANCE	COLOR YIELDED BY 100 $\mu\text{g}$ IN TERMS OF $\mu\text{g}$ ETHANOL
Oxalacetic acid	3
$\alpha$ Glycero-phosphate	20
Glyceraldehyde	1
n Propyl alcohol	22 (blue color)
n Butyl alcohol	20 (purple color)
Isobutyl alcohol	2
Allyl alcohol	10 (red color)
n Amyl alcohol	6
Isoamyl alcohol	6

In many instances the first distillation procedure could be omitted. It was found, however, that lactic acid interfered and that this interference was quite variable. After several attempts, the only procedure found effective in eliminating lactic acid interference was the preliminary distillation from basic solution.

A good portion of this error undoubtedly is dependent on the accuracy with which the second distillate is measured (the second distillates were collected and the volume measured in graduated centrifuge tubes) and on the variation in the Klett colorimeter tubes used (standardized to  $\pm 3$  Klett scale readings).

*Determination of Ethanol in Blood*—The blood level of ethanol important from the medicolegal aspect centers around 2 mg per cubic centimeter. Ethanol was added to a concentration of 1.00 mg per cubic centimeter to samples of whole citrated blood from ten individuals. One-tenth cubic centimeter of the blood-alcohol mixture was diluted to 5.00 cubic centimeters. Four and one-half cubic centimeters of  $N/12$   $H_2SO_4$  and 0.50 cc of 10 per cent  $Na_2WO_4 \cdot 2H_2O$  were added, making a total volume of 10.00 cubic centimeters. The ethanol in 1.00 cc of the supernatant was determined by the procedure described. To eliminate error arising from variation in the colorimeter tubes, a single tube was used in the blood determinations for all colorimeter readings. Blood samples analyzed after standing overnight gave the same results as those analyzed immediately after the addition of ethanol. The average recovery for forty-two determinations on these samples was 98.6 per cent. The standard error of a single determination was  $\pm 3$  per cent, indicating that nineteen times out of twenty the value obtained from a single determination will be within  $\pm 6$  per cent of the true value.

Without preliminary precipitation of blood proteins and erythrocytes with tungstic acid, very high blank readings were obtained, presumably due partially or totally to acetaldehyde in the erythrocytes.<sup>16</sup> Similarly, high blanks were obtained if the first distillation was omitted.

Values for the normal ethanol content of blood reported in the literature range from 4 to 40  $\mu g$  per cubic centimeter<sup>1, 3, 17</sup> and those for interfering substances estimated as ethanol have been reported as 40  $\mu g$  per cubic centimeter.<sup>18</sup> In our experiments blank determinations (without added ethanol) on blood samples from thirteen normal individuals gave values from 1 to 10  $\mu g$  per cubic centimeter, with an average of 6  $\mu g$  per cubic centimeter. There is no assurance that these values represent the true normal ethanol level since this method of determination is not absolutely specific. Such levels obviously do not interfere in the determination of ethanol concentrations in blood important from a medicolegal aspect.

The extreme sensitivity of this method makes it possible to do blood alcohols on the amount of blood obtainable from finger puncture. The collection and initial dilution of the blood could be carried out with a white blood cell dilution pipette (giving a dilution of 1:20). Before this could be accepted from a medicolegal aspect it would be necessary to ascertain whether the blood alcohol level is the same in blood obtained by finger puncture as in the blood obtained by venipuncture.

It is noteworthy that methanol and acetone do not interfere in this test.

#### SUMMARY

A method for the determination of ethanol in blood and other biologic fluids is described based on oxidation of the alcohol to acetaldehyde with subsequent determination of the latter photocolometrically with p-hydroxydiphenyl reagent. This method is more sensitive than pre-existing methods and has a precision of approximately  $\pm 6$  per cent on a single blood determination.

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# THE MICROCOLORIMETRIC DETERMINATION OF SODIUM IN HUMAN BIOLOGIC FLUIDS

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BARBER and Kolthoff<sup>1</sup> in 1928 described the use of the triple salt, uranyl zinc sodium acetate, for the gravimetric determination of sodium. Subsequently Butler and Tuthill<sup>2</sup> applied this method with modifications to the determination of sodium in biologic products. Although the gravimetric procedure gives good results, its operation is tedious and requires large amounts of often scarce biologic material. More recently one or both of these shortcomings have been overcome in part by treatment of the precipitate by various colorimetric<sup>3</sup> or titrimetric<sup>4</sup> reactions. In using these more rapid but also more complex techniques, we gained the impression that greater ease of operation would be achieved if the microquantities of sodium contained in 0.2 cc of serum or plasma could be measured in terms of the yellow color of the triple salt complex. Examination of the literature showed that in 1929 Caley and Foulk<sup>5</sup> had applied this principle to inorganic samples with limited success. Apparently the large amounts of material requisite for the procedure and the difficulties embodied in measuring the density of the resultant yellow color by the then used optical colorimeters discouraged the further application of this ideally simple procedure to inorganic materials<sup>6</sup> and biologic specimens. The excellent results which we obtained in preliminary tests of this principle in which the Klett-Summerson photoelectric colorimeter was employed for measuring the density of the yellow color of solutions containing varying amounts of the sodium zinc uranyl acetate complex prompted us to attempt the adaptation of this technique to the determination of sodium in biologic fluids. The analytic adequacy of the method which was evolved as the result of this effort was amply shown by recovery and reproducibility tests. The concordant results obtained with this rapid technique when employing 0.2 cc samples of urine, cerebrospinal fluid, and various blood fractions recommends its use for pediatric studies.

## EXPERIMENTAL

### *Reagents —*

Uranyl zinc acetate<sup>7</sup> 10 Gm of uranyl acetate were dissolved in 50 cc of boiling water containing 2 cc of glacial acetic acid. In another flask 30 Gm of zinc acetate were dissolved in 50 cc of boiling water containing 1 cc of glacial acetic acid. The boiling solutions were mixed and again heated just to boiling. After standing overnight at room temperature, the resultant solution was filtered by gravity and mixed with an equal volume of 95 per cent ethanol.

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This solution was refrigerated at 4° C for forty eight hours and then filtered by gravity for use. The reagent is stable at room temperature.

Ethanol 95 per cent by volume

Trichloroacetic acid 20 per cent solution

Sodium standard 508.4 mg of oven dried (100° C) sodium chloride were dissolved in 100 cc of triple distilled water, 1 cc of this solution is equivalent to 2 mg of sodium.

#### DETAILS OF PROCEDURE

In order to determine the colorimeter filter best suited for the photoelectric measurement of the yellow color of sodium uranyl zinc acetate the spectral transmission of solutions of the salt equivalent to 1 mg of sodium was measured in a Coleman Universal spectrophotometer, Model 11. These data indicated that

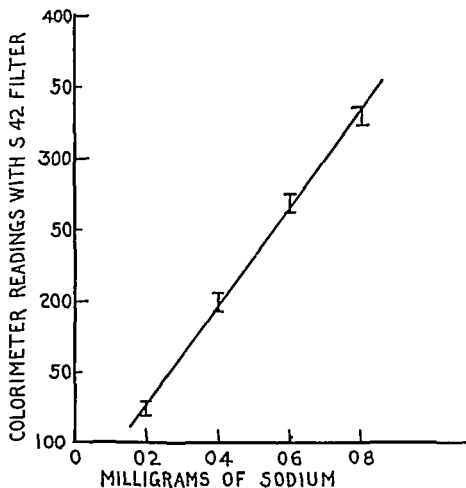


Fig. 1—Relation of color intensity to amount of sodium. Each point represents the average of five determinations. The bars above and below each point indicate the deviation range of the readings.

maximum optical density for this colored solution occurs in the region 420 to 440  $\mu$  and that to realize maximum sensitivity for the test the readings should be made with filter S-42.

The analytical efficacy of the scheme was ascertained as follows. Suitable aliquots (0.1 to 0.4 cc) of the standard in 15 cc graduated centrifuge tubes were each treated with 1 cc of the reagent. After refrigeration for one hour at 4° C the samples were centrifuged and the supernatant fluid decanted and discarded. The yellow precipitate was washed by centrifugation with 2 cc of 95 per cent ethanol and dissolved in 5 cc of water. These solutions were transferred to the colorimeter tubes and their density measured in the Klett



Summerson photoelectric colorimeter using filter S-42. The results of this experiment, which are shown in Fig 1, demonstrate that the color intensity of the solution varies in accordance with Beer's law for this range of sodium concentration. This color was found to be stable for several hours.

The deviations of the color readings from the mean indicate that the experimental error of the method is approximately  $\pm 5$  per cent, which is no greater, if not less, than that incurred in the gravimetric, titimetric, or other colorimetric methods.

The adequacy of the one hour of refrigeration and the single alcohol washing employed in these tests was demonstrated by the concordance of the readings of samples of the standard so obtained with those of similar samples which were allowed to stand in the refrigerator overnight and which were washed three times with 95 per cent ethanol.

Since the application of the method to urine was expected to entail the removal of phosphate ions, solutions of  $\text{KH}_2\text{PO}_4$  which contained the interfering ions in more than twice their normal concentration in the urine were treated in the manner described for the sodium standard. This test disclosed that the precipitate obtained under these conditions, unlike the sodium acetate complex, was insoluble in water, could be removed by centrifugation, and did not contribute any measurable color to the supernatant fluid. This observation indicated that the urinary phosphates could be readily disposed of in this manner rather than by the use of supplementary reagents.<sup>2</sup> The analytical adequacy of removing phosphate ions by this scheme was further shown by the agreement of sodium values of 0.2 and 0.3 cc aliquots of the standard before and after the addition of 2 mg of  $\text{KH}_2\text{PO}_4$  (Table I). This amount of phosphate ion is equivalent to approximately 5 Gm of this ion per liter or about twice its normal concentration in human urine.

#### APPLICATION OF METHOD TO BIOLOGIC MATERIALS

In applying the technique to urine derived from adults and children the following procedure was found satisfactory: to 0.2 cc of urine in a 15 cc centrifuge tube was added, with mixing, 1 cc of reagent. After refrigeration for one hour at  $4^\circ \text{C}$  the mixture was centrifuged at 3,000 revolutions per minute for ten minutes. The supernatant fluid was discarded and the tubes were carefully drained by inversion. The yellow precipitate was resuspended in 2 cc of 95 per cent alcohol, washed by centrifugation, and again drained by inversion. The precipitate was dissolved in 5 cc of distilled water. (The turbidity which may result from the presence of an excess of phosphate ions is easily removed by centrifugation.) The clear yellow supernatant fluid was decanted into the colorimeter tubes and the intensity of the yellow color measured in the Klett-Summerson colorimeter using an S-42 filter. A parallel determination was also done on a 0.2 cc aliquot of the standard. The sodium content per cubic centimeter of urine was obtained from the following formula:

$$\text{Reading of unknown} / \text{reading of standard} \times 0.4 \text{ mg of Na} \times 5$$

It is obvious that careless handling of the tubes during the washing processes will result in the loss of precipitate which will lead to serious analytic errors. For this reason all determinations should be done in duplicate.

The sodium content of spinal fluid also can be determined directly in 0.2 cc samples as described for urine. The presence of the small amounts of phosphate ions and protein which usually occur in this material does not interfere with this assay.

TABLE I RECOVERY OF ADDED SODIUM

SAMPLE	SODIUM ADDED (MG)	SODIUM FOUND (MG)	RECOVERY OF ADDED SODIUM (%)
KH <sub>2</sub> PO <sub>4</sub> , 2 mg	0	0	
KH <sub>2</sub> PO <sub>4</sub> , 2 mg	0.4	0.4	100
KH <sub>2</sub> PO <sub>4</sub> , 2 mg	0.6	0.6	100
Urine P, 0.2 cc	0	0.129	
Urine P, 0.2 cc	0.2	0.327	99
Urine G, 0.2 cc	0	0.557	
Urine G, 0.2 cc	0.2	0.748	95.4
Urine M, 0.2 cc	0	0.473	
Urine M, 0.2 cc	0.2	0.668	97.5
Serum D, 0.1 cc	0	0.315	
Serum D, 0.1 cc	0.2	0.520	102
Serum W, 0.1 cc	0	0.308	
Serum W, 0.1 cc	0.2	0.515	103
Plasma C, 0.1 cc	0	0.362	
Plasma C, 0.1 cc	0.2	0.562	100

In the analysis of serums, plasmas, or whole blood 0.2 cc aliquots are treated with 0.6 cc of 20 per cent trichloroacetic acid and, after centrifugation 0.4 cc of the supernatant fluid (equivalent to 0.1 cc of the original sample) is treated with 1 cc of the reagent and the determination completed as described for urine. In practice whenever sufficient material is available it is more convenient for duplicate analyses to obtain two 0.4 cc aliquots from 0.5

TABLE II SODIUM CONTENT OF VARIOUS HUMAN BIOLOGIC FLUIDS

SUBJECT	SAMPLE	SODIUM CONTENT
C, Adult, male	Serum	316 mg %
E, Adult female	Plasma	307 mg %
H, Adult male	Whole blood	224 mg %
J, Infant female	Serum	325 mg %
W, Infant, male	Cerebrospinal fluid	285 mg %
D, Infant female	Cerebrospinal fluid	312 mg %
V, Infant female	Cerebrospinal fluid	294 mg %
J, Premature infant, male	Urine	129 Gm/day
G, Infant male	Urine	314 Gm/day
S, Infant male	Urine	550 Gm/day
A, Adult male	Urine	175 Gm/day
M, Adult female	Urine	137 Gm/day

cc portions of the blood fractions to which are added 1.5 cc of the trichloroacetic acid solution. The adequacy of the technique as applied to these biologic fluids is indicated by the quantitative recovery of added sodium (Table I).

The results of analyses of biologic fluids obtained from human subjects of various ages are given in Table II. It will be noted that these are in the range of the values reported by others.

## SUMMARY

A rapid microcolorimetric procedure for the estimation of the sodium content of biologic fluids has been described which yields values comparable to those of methods requiring larger amounts of material and greater expenditure of time

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## A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF PREGNANDIOL

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THE earliest methods for the quantitative determination of pregnandiol were gravimetric. Some years later Talbot and co-workers<sup>1</sup> described a colorimetric technique—the addition of sulfuric acid to pregnandiol produced a color with a maximum absorption at 420 millimicrons. The accuracy of the method was reported to be within 3 per cent for quantities of pregnandiol between 0.05 and 0.3 milligrams. The relationship between color density and quantity of pregnandiol was according to the equation  $C = 2 - \frac{\log C}{K}$ . Although this reaction was entirely satisfactory in the hands of Talbot and co-workers<sup>1</sup> others<sup>2,3</sup> found that it shared some of the well known unpleasant habits of sulfuric acid colors in general. The Liebermann-Burchardt reaction for instance, has a tendency to develop adventitious tints which may be most disturbing. Other colorimetric methods using sulfuric acid often have been replaced for the same reason. Guterman<sup>3</sup> found spurious colors so troublesome during his pregnandiol determinations that it became necessary for him to modify his technique considerably and to prepare fresh reagents every two to three days.

In view of these facts an attempt was made to develop another color reaction which would be at least as sensitive as the sulfuric acid method yet which would not develop adventitious colors. Of a number of reactions tested one in particular gave indications of being suitable. It has been reported<sup>4</sup> that cholesterol produces a characteristic color when added to acetyl chloride and zinc chloride in a solution of glacial acetic acid. Subsequent workers<sup>5</sup> developed this reaction into a quantitative method for the determination of blood cholesterol. In our experience it has distinct advantages over the usual Blood method. Despite certain theoretic predictions it was found that pregnandiol gives a stable quantitative color reaction with acetyl chloride and zinc chloride within certain limits the quantity of pregnandiol and the color intensity have a straight line relationship so that direct colorimetric readings can be made.

### EXPERIMENTAL

Carefully recrystallized pregnandiol was used throughout. Ether alcohol solutions containing 0.10 and 1.0 mg. per milliliter were prepared; the desired quantity was pipetted into the reaction vessel and the solvent evaporated. The

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zinc chloride reagent was prepared by dissolving 38 Gm of pure zinc chloride in sufficient glacial acetic acid to make a final volume of 100 milliliters

The absorption spectrum of the color complex, after development for thirty minutes at 50° C, is shown in Fig 1. Maximum absorption occurred between 370 and 450 millimicrons. At wave lengths below 300 m $\mu$  the solvent became too opaque for further determinations.

Various factors which might affect the color development, such as proportion of reagents, temperature, time, and concentration, were studied.

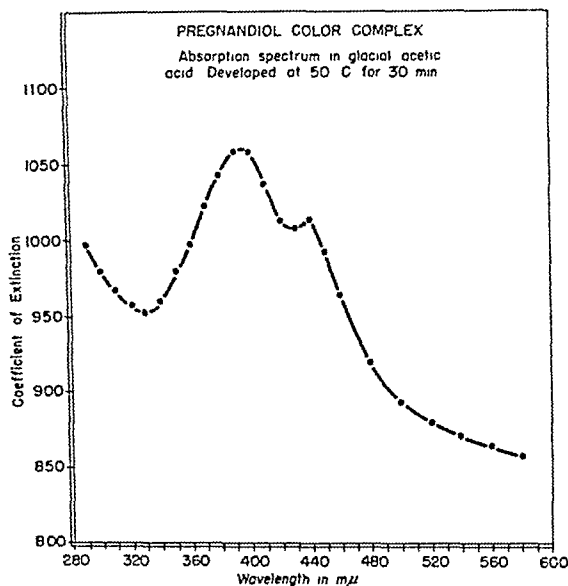


Fig 1

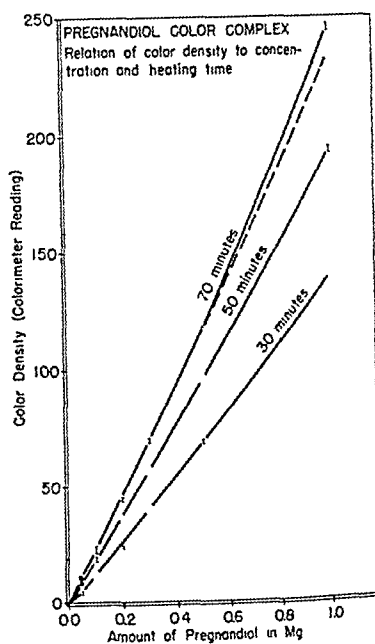


Fig 2

It was found that 25 cc of acetyl chloride were most suitable to a final volume of 10 cc attained by the addition of zinc chloride reagent.

The highest temperature at which color development could be carried out without substantial losses of acetyl chloride by volatilization was found to be 50° C.

Color development at a constant temperature of 50 ± 2° C proceeds rapidly during the first fifty minutes. Small quantities of pregnandiol (less than 0.2 mg) attain a constant color density by 150 minutes, but with larger amounts (0.3 to 1.0 mg) the color continues to increase in density even after 300 minutes of incubation. When cooled to room temperature the color is stable and remains constant for at least two and one-half hours.

A different aspect of the relationship between color density, concentration and incubation time is shown in Fig 2. The data are taken from runs carried out in triplicate. It will be seen that color versus concentration is a straight line relationship if the heating time is thirty to fifty minutes, incubation for longer than sixty minutes yields aberrant results with higher concentrations of pregnandiol.

It was found impracticable to control the temperature and the heating so closely that the use of a standard solution became unnecessary. With the use of a standard (0.5 mg pregnandiol) the method was found to be accurate within 4 per cent.

#### PROCEDURE

The unknown quantity of pregnandiol is dissolved in a suitable amount of solvent, such as ether alcohol, and an aliquot representing approximately 0.1 to 1.0 mg is pipetted into a 10 c.c. glass stoppered volumetric flask. The solvent is evaporated off. Approximately 6 c.c. of zinc chloride reagent are placed in the flask and 2.5 c.c. of acetyl chloride are added from a small burette. A standard for comparison, representing 0.50 mg of pregnandiol, is prepared in similar fashion. The flasks are incubated in a hot water bath at 50° C for thirty minutes. The flasks are then dipped briefly in ice water until cool and are allowed to stand at room temperature for twenty minutes. The volume is adjusted to 10 c.c. with zinc chloride reagent. After careful mixing of the solution to insure color uniformity, it is ready for reading.

#### SUMMARY

A new colorimetric reaction with pregnandiol, based on its interaction with acetyl chloride and zinc chloride in glacial acetic acid solution is described. The characteristics of the reaction and the technical procedure are discussed briefly.

We express our thanks to Dr. W. J. Dann of the Department of Biochemistry for the spectrophotometric determinations.

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# THE EFFECT OF CHLORINE ON COMPLEMENT FIXATION

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MANY laboratories are equipped with continuous-flow water stills which permit considerable quantities of chlorine in the tap water to be condensed in the distillate. In our experience with a Bainstad "Q" type of still, the chlorine content of the distillate may be as high as the water from which it is prepared, and may, therefore, fluctuate in relation to the chlorine residual of the undistilled water.

When this still was first put into use the distillate was used in preparing saline for the Kolmer-Wassermann test and we were not able to get a complement titration in the approved dilution range even though all the biologic reagents had been satisfactory when titrated with saline of distillates from other stills. This quality of interfering with the complement titrations was found to correlate with the chlorine residual of the various distillates.

Directions for making the saline for Kolmer's modification of the Wassermann test specify the use of 0.85 per cent sodium chloride. The addition of 10 cc of 10 per cent magnesium sulfate to each liter is recommended but is not always essential. Heating in an Arnold sterilizer for one hour is considered advisable if the saline is not used immediately.<sup>1, 2</sup> No specific criteria of acceptable water are given in reference to chlorine residual.

*High Chlorine Residual (Above 0.8 Parts Per Million)*—When the chlorine residual of the water is 0.8 parts per million or higher, complete hemolysis is not obtained in any tubes of the hemolytic titration using Kolmer's method. Sheep cell suspensions using saline with these high chlorine residuals show a slight change in color, appearing darker and somewhat brown. At times even lower chlorine residuals will prevent complete hemolysis in all of the titration tubes.

*Study of Lower Chlorine Residuals (0.6 Parts Per Million or Less)*—The total chlorine residual of freshly distilled water was determined with a comparator using the orthotoluidine method.<sup>3</sup> Chlorine free water (Table I, A) was obtained by autoclaving a portion of the water for twenty minutes at 15 pounds, once or twice as needed. Water containing three intermediate amounts of chlorine (B, C, and D) was prepared by mixing the chlorine-free (A) and the chlorine containing (E) water.

Five 0.85 per cent saline solutions with magnesium sulfate added as recommended by Kolmer, were prepared using distilled water A, B, C, D, and E (as in the preceding paragraph). All reagents for use in the Kolmer-Wassermann test were prepared with the five saline solutions and five titrations and quantitative tests were set up. In the first set of experiments five portions of

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sheep cells were washed and packed separately with the five saline solutions of varying chlorine residuals. For the second set of experiments the sheep cells were washed, packed, and diluted to 20 per cent with chlorine free saline, and five portions were further diluted to 2 per cent with the varying salines. The same Kolmer antigen and hemolysin were used in both experiments, while the Lyovac complement and positive sera were different.

The results of the ten titrations and quantitative tests are shown in Table I. It is to be noted that with each increase in chlorine residual more hemolysin or more complement or more of both is required for the tests as indicated by the titration.

TABLE I

EXPERIMENT	SOLUTION	CHLORINE RESIDUAL (P.P.M.)	HEMOLYSIN TITRATION	ACTIVE UNIT OF COMPLEMENT IN ML. OF 1:30 DILUTION	QUANTITATIVE KOLMER WASSERMAN (ML. OF POSITIVE SERUM)					
					0.2	0.1	0.05	0.025	0.0125	CONTROL 0.2
I	A	0	1:5000	0.4	4	4	4	4(3)	±(-)	-
	B	15	1:5000	0.45	4	4	4	4(3)	±(-)	-
	C	30	1:5000	0.5	4	4	4	4(2)	1(-)	-
	D	45	1:4000	0.5	4	4	4	4(2)	±(-)	-
	E	60	1:3000	0.5	4	4	4	4(1)	±(-)	-
II	A	0	1:8000	0.5	4	4	5(1)	-	-	-
	B	12	1:6000	0.5	4	4	5(1)	-	-	-
	C	25	1:5000	0.4	4	4	3(1)	-	-	-
	D	37	1:4000	0.5	4	3(1)	1(-)	-	-	-
	E	50	1:4000	0.5	4	5(1)	-	-	-	-

\*Readings made ten minutes after clearing of controls.

Reading made after one hour of incubation is shown in parenthesis if different from the earlier reading.

Quantitative tests were set up using four different positive sera on each day. The results with only one sera are shown in Table I. The results were similar with all sera, that is slightly weaker reactions with the chlorine containing saline.

#### DISCUSSION

These results indicate that saline made with chlorine free distilled water should be used for diluting reagents for complement fixation tests. The advantages are as follows:

1. Removal of one factor which may make it impossible to get a titration in the approved range.
2. Higher sensitivity of tests.
3. Lower cost of reagents: this is particularly important in reference to the complement.

It is interesting to note that the actual tests do not indicate that the chlorine has an enhanced deleterious effect on the complement during the long incubation fixation. The explanation of this is not apparent from the present observations. This study included only the Kolmer Wassermann complement



fixation test, but it seems reasonable to assume that the same effect would be seen with any complement fixation procedure

Distilled water containing chlorine may be rendered chlorine-free by several methods

- 1 Storage for one to several months
- 2 Boiling to two-thirds or one-half the original volume
- 3 Autoclaving at fifteen pounds pressure for twenty minutes once or twice as needed
- 4 Inserting an activated-carbon filter to remove the chlorine from water going to the still

Before use, the water must be tested for chlorine residual. Since a satisfactory water must be chlorine-free, there is no need to determine the content if present.

#### CONCLUSIONS

1 Attempts to titrate the reagents for the complement fixation test are unsuccessful when distilled water with a high chlorine residual (0.8 parts per million or higher) is used in preparing the saline solution.

2 Complement fixation tests using water with lower chlorine residuals (0.6 parts per million or less) require more hemolysin or more complement or more of both than tests using chlorine-free water.

3 Only chlorine-free water should be used in saline for complement fixation procedures.

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## THE DYNAMICS OF PROTEIN METABOLISM

### I THE INTERRELATIONSHIP BETWEEN PROTEIN AND CALORIC INTAKES AND THEIR INFLUENCE UPON THE UTILIZATION OF INGESTED PROTEIN FOR TISSUE SYNTHESIS BY THE ADULT PROTEIN DEPLETED RAT

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#### INTRODUCTION

OPTIMAL nutrition remains a major problem of medicine and surgery despite the accumulating knowledge concerning vitamins amino acids and other essential dietary factors. Until certain relationships are more clearly elucidated however, we still cannot utilize this knowledge with full effectiveness. Important among these is the interrelationship between the level of protein intake, the energy intake, and the utilization of dietary protein. The present studies were undertaken in order to examine this interrelationship under controlled conditions. The basic plan was to study the rate of utilization of protein for tissue fabrication by the protein depleted animal under the following circumstances: (1) varying caloric intake with constant protein intake, (2) varying protein intake with constant caloric intake, and (3) simultaneous variation of caloric and protein intakes.

In these studies we have examined not only gross rates of fabrication of protein in the body as a whole but also rates in various body compartments. This paper deals only with the gross metabolism of protein in the rat. A second paper will contain evidence which demonstrates a fundamental similarity of the protein fabricating mechanisms of the rat and man. Later papers will deal with the relative rates of protein synthesis in various body compartments of protein depleted animals.

#### MATERIALS AND METHODS

In these experiments protein depleted, young adult male albino rats (Sprague Dawley) were used. At the start of the depletion period their weights averaged 210 grams. For two and one-half months they were depleted upon a low protein diet which contained adequate offerings of calories, vitamins, minerals, and other dietary essentials. The depletion diets and regime have been described.<sup>1</sup> At the end of the depletion period the average weight of the animals was 154 grams. Animals were then individualized. Groups of five or six animals

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were selected to be fed each of the experimental diets. The animals were chosen so that each group was comparable with respect to initial weight, weight loss, serum protein concentration, and hemoglobin concentration.

The basic composition of the diets was the same in all three experiments. For the protein, a half and half mixture of bovine lactalbumin\* and casein vitamin test was used. The composition of the diets is given in Tables I and II. It is evident from Table I that in Experiment 1 the absolute quantity of protein offered per day was constant, but the quantity of carbohydrate varied. In Experiment 2 the absolute quantity of protein fed per day was varied and the total caloric value of the diet offered was maintained constant. Experiment 3 utilized the same diet throughout, offered in different amounts to different groups of animals. Diets were offered in weighed amounts each day. Diet consumption was measured daily and the experimental feeding period was fourteen days.

TABLE I COMPOSITION OF DIETS

DIET	DIET FED PER DAY (GM)	PROTEIN OFFERED PER FAT PER DAY (GM)	CALORIES OFFERED PER FAT PER DAY	INGREDIENTS PER 100 GM OF DIET (GM)							
				FIBER	CORN OIL	LACTALBUMIN	CASEIN	DETRIN	SALT MIX	LIVER CONCENTRATE	WATER
Experiment 1											
A	48	156	15	49	11.3	18.1	16.3	20.3	12.5	5.1	64
B	80	156	25	49	68	10.8	9.8	43.5	7.5	1.9	106
C	105	156	35	52	51	8.3	7.5	56.9	5.7	1.4	67
D	150	156	48	50	36	5.8	5.2	61.5	4.0	1.0	116
E	180	156	60	52	30	4.8	4.4	67.9	3.3	0.8	86
F	150	---	48	50	40	0.0	0.0	71.0	4.0	1.0	127
Experiment 2											
A	150	0.53	48	50	3.9	1.8	1.6	68.0	4.0	1.0	124
B	150	0.83	48	50	3.8	3.0	2.7	66.0	4.0	1.0	122
C	150	1.14	48	50	3.7	4.3	3.8	64.0	4.0	1.0	119
D	150	1.51	48	50	3.6	5.8	5.2	61.5	4.0	1.0	116
E	150	1.79	48	50	3.5	7.3	6.5	59.1	4.0	1.0	114
F	150	2.57	48	50	3.1	11.6	10.5	51.9	4.0	1.0	106
Experiment 3											
A	4.7	0.46	15	50	3.6	5.8	5.2	61.5	4.0	1.0	116
B	7.8	0.76	25	50	3.6	5.8	5.2	61.5	4.0	1.0	116
C	10.9	1.06	35	50	3.6	5.8	5.2	61.5	4.0	1.0	116
D	15.0	1.46	48	50	3.6	5.8	5.2	61.5	4.0	1.0	116
E	18.7	1.82	60	50	3.6	5.8	5.2	61.5	4.0	1.0	116
F	4.7	0.48	15	50	3.6	5.8	5.2	61.5	4.0	1.0	116

Initial determinations of the various blood compartments were made by methods which have been described in detail elsewhere.<sup>2</sup> At the end of the test period these values were re-determined. The animals were then sacrificed. Liver, heart, kidneys, and approximately 40 per cent of the blood were removed. The gastrointestinal tract was stripped of its content. The carcass with the residual contents, including the gastrointestinal tract, was weighed and analyzed for water, fat, protein, and ash.

\*Borden's No. 1542

†SMACO vitamin test, General Biochemicals Inc.

TABLE II DAILY VITAMIN RATION

VITAMIN	QUANTITY OFFERED PER DAY
Thiamin chloride	80 gamma
Riboflavin	120 gamma
Niacin	200 gamma
Pyridoxine HCl	90 gamma
Calcium pantothenate	190 gamma
Choline chloride	27 mg
Oleum percomorphum	
Vitamin A	60 USP units
Vitamin D	9 USP units

All diets offered the equivalent of the vitamins per animal per day except in Experiment 3. In this experiment the vitamin offering was proportional to the quantity of diet offered diet 3 D providing the standard allowance Diet 3 F otherwise identical with 3 A offered the standard quantities of the vitamins

The method of carcass analysis was as follows Each carcass was cut into pieces with a scissors and the pieces ground in a motor driven meat grinder Grinder and parts were rinsed with alcohol and wiped with a weighed filter paper Crinding washing and paper were collected in a weighed evaporating dish and dried at a temperature of 60 C in a hot air oven for a minimum of five days Following this the dish and contents were weighed and the contents were transferred to a weighed fat free mullin sack and extracted for twelve hours with alcohol in a Soxhlet extractor They were re extracted for twelve hours with ether Following this, the sack and contents were dried again for forty eight hours in the 60 C oven cooled in a desiccator and weighed Preliminary to a hung the dried fat free carcass was ground to a fine powder in a motor driven pulverizer Dry 2 Gm samples were held in a muffle furnace at 600 C for six hours cooled in a desiccator and weighed

It was found in early experiments that complete recovery of the lost weight by these protein depleted adult animals was not associated with significant changes in the ash content of the carcass and that differences of the order of magnitude found were within the limits of variation among individual animals Therefore in Experiments 2 and 3 the ash content was determined on single samples from each of the diet groups and the pooled average so obtained was used to compute the protein content of each carcass

Protein was computed by deducting the weight of the ash from that of the dry fat free carcass In order to ascertain how closely this value approximated the conventional  $N \times 6.25$  value for the estimation of the protein the following experiment was performed Nitrogen determinations were made on seven carcasses of animals of widely differing weights The nitrogen concentration of the ash free carcass was found to be  $15.7 \pm 0.05$  per cent This value is in reasonable agreement with the commonly accepted value for animal proteins of 16.0 per cent nitrogen and with the value of 15.4 per cent found by Addis and co workers<sup>3</sup>

The livers were analyzed for fat water protein and water soluble constituents by a method similar to that used for the carcasses The details will be given in a later paper All hearts and kidneys were weighed and selected one were analyzed

#### OBSERVATIONS

Table III presents the observations on body weight diet consumption carcass fat and protein Recorded in Table IV are the average of the mean weights of the animals for the fourteen day experimental period the average weight grams the calculated surface areas<sup>4</sup> the average daily protein intakes caloric intakes and rates of protein gain

The protein intakes are recorded as grams of protein ingested ( $N \times 6.25$ ) per kilogram of body weight per day The rate of protein utilization is recorded in an analogous fashion as grams of protein gained per kilogram of body weight per day Caloric intake appears as calories per square meter of body surface per day These units may appear unnecessarily devious and cumbersome

some, an inspection of the nature of the quantities, however, shows that they reduce the measured values to a system of units which is independent of the size of the animal. The advantages of this for comparing animals of different sizes within the same species or of different species are obvious and will become clearer.

TABLE III ORIGINAL DATA FOR BODY WEIGHTS, DIET CONSUMPTION, CARCASS FAT AND PROTEIN\*

DIET	NUMBER OF ANIMALS	PFEDEPLETED WEIGHT (GM)	DEPLETED WEIGHT (GM)	WEIGHT AFTER 14 DAYS REPLETION (GM)	DIET CONSUMED IN 14 DAYS (GM)	CARCASS FAT (GM)	CARCASS PROTEIN (GM)
<i>Experiment 1</i>							
A	4	211 ± 17	148 ± 25	155 ± 20	67 ± 0.2	110 ± 1.55	27.9 ± 0.26
B	5	216 ± 3.6	153 ± 2.3	181 ± 1.8	112 ± 0.1	120 ± 1.62	32.6 ± 0.21
C	5	211 ± 2.6	151 ± 3.2	200 ± 3.0	146 ± 0.4	250 ± 1.52	35.7 ± 0.21
D	5	212 ± 4.7	153 ± 3.1	207 ± 2.0	206 ± 0.9	331 ± 1.93	34.8 ± 0.26
E	5	210 ± 1.5	150 ± 2.7	215 ± 2.9	233 ± 4.0	364 ± 1.23	35.2 ± 0.13
F	4	214 ± 3.0	158 ± 3.9	146 ± 1.5	119 ± 1.7	114 ± 0.67	25.9 ± 0.76
<i>Experiment 2</i>							
A	5	205 ± 1.8	146 ± 2.8	156 ± 3.3	165 ± 5.5	192 ± 1.09	26.7 ± 0.44
B	6	208 ± 2.6	146 ± 1.5	188 ± 1.3	201 ± 1.5	278 ± 0.97	32.1 ± 0.23
C	6	208 ± 2.7	147 ± 2.1	208 ± 2.6	207 ± 0.1	290 ± 1.48	35.5 ± 0.73
D	6	206 ± 1.4	148 ± 1.8	222 ± 1.5	206 ± 0.8	309 ± 2.63	35.9 ± 0.26
E	6	207 ± 2.9	145 ± 2.7	229 ± 3.5	204 ± 0.7	301 ± 2.36	36.2 ± 0.21
F	6	207 ± 3.7	145 ± 3.4	235 ± 3.4	206 ± 0.2	261 ± 0.50	40.3 ± 0.16
G	5	208 ± 3.2	149 ± 3.8	132 ± 3.8	129 ± 3.9	105 ± 1.16	25.2 ± 0.66
<i>Experiment 3</i>							
A	4	214 ± 1.9	155 ± 2.5	159 ± 2.2	66 ± 0.0	136 ± 0.32	29.6 ± 0.14
B	5	214 ± 3.5	154 ± 2.1	181 ± 2.1	109 ± 0.2	202 ± 1.07	32.3 ± 0.21
C	5	213 ± 1.8	158 ± 2.8	204 ± 3.8	151 ± 0.3	252 ± 2.36	35.9 ± 1.06
D	5	214 ± 2.2	157 ± 3.4	229 ± 3.3	208 ± 0.6	251 ± 2.26	38.0 ± 0.13
E	5	215 ± 3.8	153 ± 4.5	240 ± 2.3	247 ± 3.2	407 ± 1.95	38.7 ± 0.23
F	5	215 ± 3.0	156 ± 3.6	158 ± 3.1	66 ± 0.2	90 ± 1.11	30.3 ± 0.68
G	5	213 ± 3.4	155 ± 1.9	135 ± 1.7	111 ± 4.2	132 ± 1.01	25.2 ± 0.47

\*The values given are means and standard errors for animals fed each diet. Standard errors computed by  $SE = \sqrt{\frac{\sum (x-m)^2}{n(n-1)}}$

Protein gains were measured from an initial value for carcass protein estimated in the following manner. In each experiment a control group of depleted animals was maintained on the low protein ration through the experimental period. This group was sacrificed with the others. The mean quantity of protein found in the carcasses of these animals was subtracted from the quantity determined in each of the repletion groups. The value thus obtained included the quantity of protein lost by the control group, that is, the wear and tear protein loss. This quantity we have determined and subtracted from the apparent gains of the animals in the repletion groups in order to obtain the measure of protein fabrication over and above that which merely replaced the wear and tear losses.

In order to include all, or practically all, of the protein fabricated in the experimental interval, the increase in the liver protein was added to that in the carcass protein. Increases in other compartments not included in the car-

TABLE IV WEIGHT GAINS AND RATIO OF PROTEIN UTILIZATION AT VARIOUS CALORIC AND PROTEIN INTAKE LEVELS

NUMBER OF ANIMALS	AVERAGE MEAN WEIGHT FOR 14 DAYS* (GM)	AVERAGE WEIGHT GAIN FOR 14 DAYS (GM)	SURFACE AREA AVERAGE FOR 14 DAYS (M <sup>2</sup> )	DAILY PROTEIN INTAKE (GM/KG)	DAILY CALORIC INTAKE (CAL/M)	DAILY PROTEIN GAIN (GM/KG)
<i>Experiment 1</i>						
4	159	6	0.26	9.6	560	0.3
5	168	34	0.27	8.9	900	2.3
5	177	61	0.28	8.6	1240	3.5
5	182	67	0.28	8.3	1670	3.1
5	187	76	0.29	7.3	1840	3.2
4	153	-18	0.26	0.4	1060	—
<i>Experiment 2</i>						
5	154	13	0.26	2.8	1510	0.0
6	168	41	0.27	4.8	1600	2.6
6	178	61	0.28	6.3	1690	3.7
6	186	74	0.29	8.0	1640	3.7
6	188	85	0.29	9.0	1610	4.6
6	191	90	0.29	14.8	1610	5.0
5	140	-71	0.24	0.5	1210	—
<i>Experiment 3</i>						
5	157†	2	0.26	3.4	510	1.6
4	154	3	0.26	2.9	580	1.4
5	162	28	0.27	4.6	930	2.0
5	179	50	0.28	5.9	1220	3.8
5	193	72	0.30	7.5	1610	4.4
5	197	87	0.30	8.7	1880	4.5
5	145	-20	0.25	0.4	1040	—

Mean weight is the average of the initial and final weights of the animals in each group for the fourteen day period

†High vitamin diet.

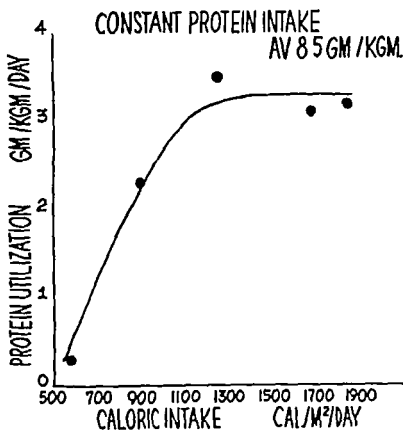


Fig 1—The influence of level of caloric intake on protein utilization

CONSTANT CALORIC INTAKE  
AV 1630 CAL /M<sup>2</sup>/DAY

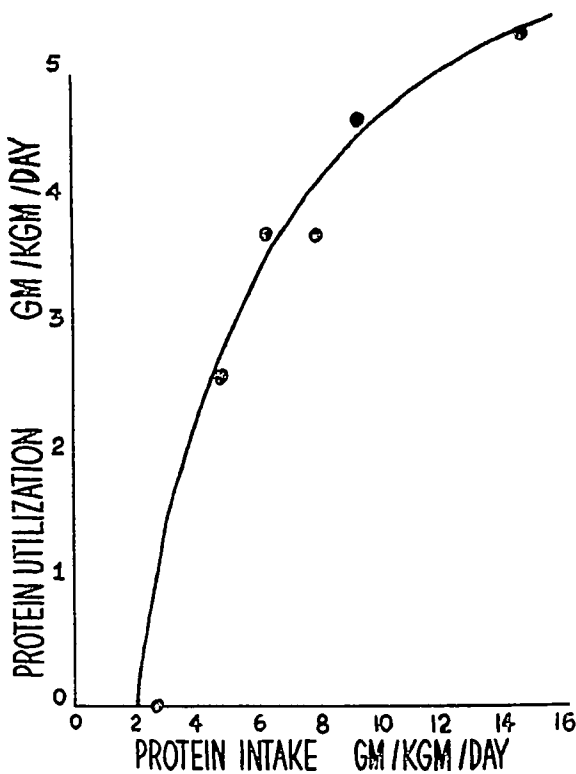


Fig 2 —The influence of level of protein intake on protein utilization

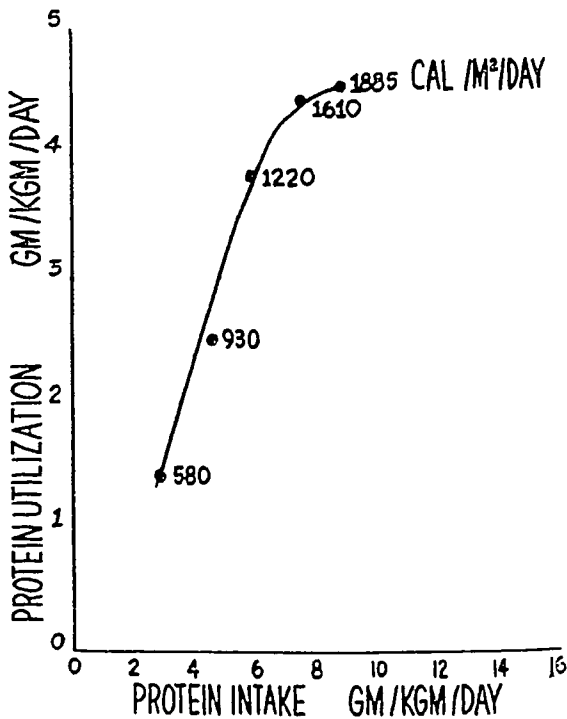


Fig 3 —The influence of simultaneous variation of protein and caloric intake levels on protein utilization

cases were not of sufficient magnitude to necessitate their addition. Recorded rates of gain therefore represent for practical purposes all of the protein fabricated into body tissue, exclusive of that used for maintenance.

Fig 1 demonstrates the influence of variation in caloric intake upon protein utilization, the protein intake remaining approximately constant. At the lowest level of caloric intake (560 Cal per square meter per day) protein utilization was very poor (0.3 Gm per kilogram per day). With increasing caloric intake, protein utilization rose until a maximum was reached at an intake level of 1,240 Cal per square meter per day. Beyond this point there was no additional utilization of protein despite the fact that the caloric intake increased up to 1,840 Cal per square meter per day.

The effect of varying the protein intake at a constant caloric intake is demonstrated by Fig 2. As the protein intake was raised from 2.8 to 14.8 Gm per kilogram per day, protein utilization rose. The rise was almost linear up to about 6 Gm per kilogram per day. Beyond this point the rate of protein utilization became progressively less. Nevertheless at the highest level of intake the ceiling of utilization had not yet been reached.

What occurred when increasing quantities of the same diet were fed to the depleted animals is shown in Fig 3. In this situation there is a constant ratio of protein to energy in the diet, the levels of protein and caloric intake varying

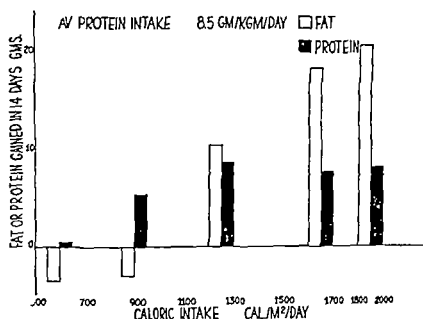


Fig 4 —The influence of caloric intake at a constant protein intake on the rate of fabrication of carcass fat and protein by depleted rats

together. Under these circumstances the rate of protein utilization increased with increasing diet intake. Looked at from another point of view when the quantity of diet was restricted the rate of protein utilization was restricted. It is evident from the data that the limitation in the rate of protein utilization was not the summation of the effects of the restriction of both protein and energy intakes and the restriction of utilization at any point was no greater than the maximum restriction induced by the restriction of the protein component alone.



CONSTANT CALORIC INTAKE  
AV 1630 CAL /M<sup>2</sup>/DAY

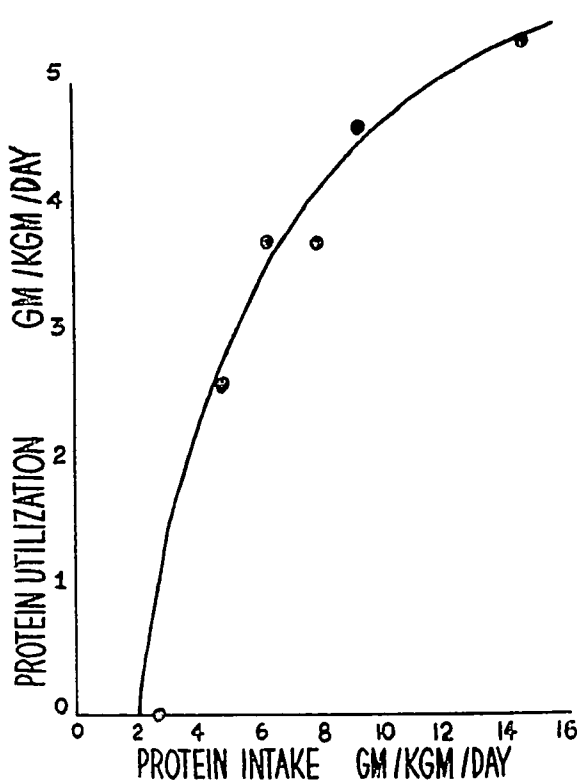


Fig 2 —The influence of level of protein intake on protein utilization

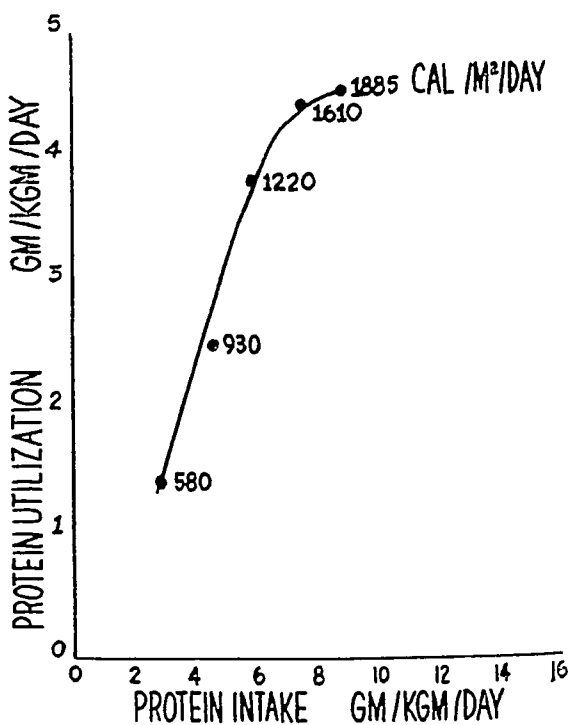


Fig 3 —The influence of simultaneous variation of protein and caloric intake levels on protein utilization

Our observations on protein depleted animals are in agreement with those cited on patients and growing animals. Thus there is a restriction in the utilization of a constant quantity of protein when the caloric intake falls below a critical value of about 1,240 Cal per square meter per day. Furthermore, elevation of the caloric intake above this level does not increase the effectiveness of the dietary protein for the synthesis of new tissue. It is of interest in this connection that the critical caloric value observed by Bosshardt and co workers<sup>8</sup> with the *growing* rat was approximately 1,250 Cal per square meter per day.

What is the significance of this critical caloric value? It is possible to elucidate the nature of the composition of this 1,240 Cal per square meter per day by breaking it down into its component parts: (a) energy used for maintenance, (b) energy for physical work, (c) energy for tissue fabrication, (d) energy stored in the form of new tissue (that is as fat and protein) and (e) waste energy in compounds excreted but not completely burned. From carcass analysis data and food intake energy balances can be constructed. Thus the animals receiving a total energy allowance of 1,240 Cal per square meter per day stored 323 Cal per square meter per day as new tissue. The difference between the total intake and the energy appearing as new tissue represents the quantity consumed for energy and wasted. Therefore the animals must have consumed and wasted a total of 917 Cal per square meter per day. From Table III it is evident that the animals which received 560 Cal per square meter per day were able just to maintain themselves during the fourteen day period. It appears justifiable therefore to take this as an approximation to the minimal maintenance energy requirements. The difference between the total energy consumed (917 Cal per square meter per day) and the energy used for maintenance (560 Cal per square meter per day) represents the energy used to construct new tissue plus waste energy, in this case 457 Cal per square meter per day.

The quantity 1,240 Cal per square meter per day therefore appears to represent the energy allowance which will supply the maintenance needs of the animal with sufficient excess to allow for maximal utilization of ingested protein for tissue synthesis. At caloric intakes below the critical level the animal must resort to burning protein for purely energy purposes. As a consequence the restriction of caloric intake below the critical point has the same net effect as a restriction of the protein intake.

Fat and protein synthesis in the depleted animal are partly independent. At low caloric intake levels both fat and protein storage are proportional to the caloric intake. At caloric intake levels above the minimum necessary for maximum protein fabrication, fat and protein metabolism are essentially independent (Fig. 4). A partial independence of the fat and protein metabolism has been observed in human beings.<sup>10</sup> It was found that obese individuals on protein intakes of 1 to 1.5 Gm per kilogram per day and on caloric intakes 30 per cent less than estimated basal needs lost weight but remained in nitrogen equilibrium. Nonobese individuals on such a regime not only lost weight, but also had negative nitrogen balances. Thus it appears that the normal organism requires a certain quantity of fatty tissue in addition to nonfat tissue, but excessive quan-

ties of fat are not only unnecessary but also probably undesirable and can be dispensed with without sacrificing more needed tissues. Some fat gain is necessary in the rehabilitation of depleted individuals for the purpose of building up energy reserves. Excessive fat gain is probably to be avoided since it appears to do little but place an extra physical strain upon the organism and in addition is economically wasteful. Therefore, *adequate* but not excessive caloric intake is the aim in convalescent and rehabilitation feeding.

The statement has been made that nitrogen *balance* can be maintained on a low caloric intake for short periods of time by giving relatively large amounts of protein.<sup>9</sup> Our findings agree with this. Thus at a low caloric intake (560 Cal

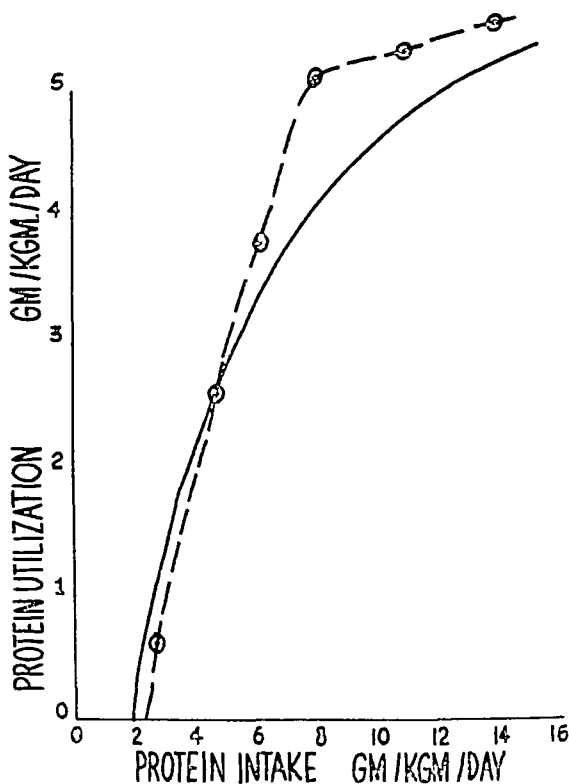


Fig. 6—The rate of protein utilization for tissue synthesis at different levels of protein intake — Adult depleted rats -- growing rats after Barnes and coworkers<sup>11</sup>

per square meter per day) and a protein intake of 9 Gm per kilogram per day very low degrees of nitrogen utilization were found, similar to those obtained by Elman and associates<sup>9</sup> in dogs. But it is obvious from our observations that this is a highly inefficient procedure in terms of nitrogen utilization. Moreover, in a debilitated patient the desired result is not the achievement of mere nitrogen equilibrium, but rather the high degrees of nitrogen retention which go with the fabrication of significant quantities of new tissue.

Long ago it was found that the rate of gain of body weight in growing animals increases as the protein intake is increased.<sup>11</sup> Forbes and co-workers<sup>1</sup> investigated this problem further with careful studies of nitrogen and energy

metabolism. Unfortunately, their lowest protein intake level was 7 Gm per kilo gram per day so that they have no data on the important lower levels of intake. More recently, this problem has been reinvestigated by Barnes and colleagues.<sup>13</sup> These investigators fed protein at various intake levels to growing rats, keeping the energy intake approximately constant. They did nitrogen determinations on the carcasses. Their data, recalculated and plotted in Fig. 6 along with the observations from Experiment 2 described here, are in excellent agreement with ours derived from the adult protein depleted rat.

This agreement becomes all the more significant when one considers the fact that the animals used in the two experiments differed both in age and size, one group being protein depleted. A fundamental relationship appears to exist between the intake level of protein and the rate of protein utilization for the fabrication of tissue in the growing animal and in the adult protein depleted animal undergoing repletion. Putting all of the foregoing facts together, we infer that the fabrication of a kilogram of new tissue in a growing animal and the reconstruction of a kilogram of tissue in an animal which has lost tissue due to protein deficiency demand similar quantities of structural material and similar constructing energies.

This does not imply that the quantitative requirements and the behavior of tissue synthesis in relation to protein intake are the same under all conditions, because certain things may modify the rate of utilization of protein. Thus, as an animal approaches its growth maximum, the rate of utilization of protein for synthesis of new tissue declines. In the face of injuries such as burns, fractures, or infections, there also is a marked decrease in the rate of tissue synthesis.<sup>14</sup> But, other things being equal, the rate of utilization of a high quality protein for synthesis of new tissue increases with increasing levels of protein intake, providing the caloric intake is adequate to supply both the basal energy needs of the organism and the energy needed for synthesis, including that stored in new tissue.

The question arises as to how far these data derived from the rat are applicable to human beings. In a later paper, evidence will be presented demonstrating the fundamental similarity of the protein fabricating mechanisms in these two groups of mammals.

#### SUMMARY AND CONCLUSIONS

Experiments are described which were designed to elucidate the interrelationships between protein intake and energy intake as they affect the utilization of protein for tissue synthesis in standard protein depleted rats.

The following conclusions are drawn:

Restriction of the caloric intake below a certain critical level restricts the utilization of ingested protein for the fabrication of tissue. Furthermore, an increase in caloric intake above this critical level does not augment the rate of utilization of a given quantity of protein above the maximum attainable with the particular protein fed under the particular circumstances of feeding (that is, level of protein intake, needs of animal, and so on). This critical level ap

pears to be approximately 1,240 Cal per square meter per day, and constitutes the energy necessary to cover the needs for maintenance, storage, tissue synthesis, and waste

Increasing the caloric intake above the critical level needed for maximal rates of protein synthesis results in increased rates of body weight gain due largely to deposition of fat

With an adequate caloric intake the rate of utilization of protein is a function of the level of intake and, in general, utilization rises with increasing intake

The fabrication of a kilogram of tissue in a growing rat and the reconstruction of a kilogram of tissue in the adult protein-depleted rat demand the same quantities of structural material and similar constructing energies

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## THE DYNAMICS OF PROTEIN METABOLISM

### II THE RELATIONSHIP BETWEEN THE LEVEL OF PROTEIN INTAKE AND THE RATE OF PROTEIN UTILIZATION BY PROTEIN DEPLETED MEN AND RATS

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#### INTRODUCTION

TODAY, perhaps as never before, there is urgent need of more knowledge concerning basic principles of nutrition. Speedy rehabilitation of underfed persons with only limited quantities of available food demands the most judicious use of the food supply. To accomplish this we must know what part the major components of the diet, principally protein and energy, play in the fabrication of body tissue.

In a previous paper<sup>1</sup> we examined the effects of varying caloric and protein intakes upon the utilization of dietary protein for tissue synthesis by protein depleted and growing rats. It was demonstrated that on an otherwise adequate diet a certain minimum level of caloric intake at a given protein intake level was needed to insure maximum tissue synthesis. Above this minimum level, increases in the caloric intake did not further augment protein utilization. Furthermore with an adequate caloric intake the rate of protein utilization increased with increasing protein intake. These relationships were found to be similar in the growing rat and in the adult depleted rat during repletion. Such observations led us to examine data from the literature on human beings and to compare them with our data on rats. After doing so, it was found necessary to extend the rat experiments to include protein intake ranges commonly used and feasible in human feeding.

The following evidence demonstrates that animal experiments can be used as qualitative and quantitative guides in elucidating the problems of human nutrition providing the appropriate common denominators are employed in making the extrapolations from one species to another. Furthermore with the combined data available on men and animals it is possible to attack intelligently the problem of constructing more efficient diets for starved and convalescing human beings.

#### METHODS AND MATERIALS

Observations on human beings providing simultaneous data on body weight, height, caloric intake, protein intake, protein source, nitrogen retention and the degree of weight loss were sought from the literature. Although most such studies deal with diseased indi-

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individuals, one study was found<sup>2</sup> which dealt with the rehabilitation of otherwise healthy individuals suffering from severe inanition. Many otherwise good data had to be discarded because one or more important measurements were absent, particularly those of body weight and height. Moreover, many reports had very brief and incomplete accounts of diet composition.

Before we could compare the observations on rats with those on men it was necessary to have some common units of measurement. Obviously one could not compare directly the daily nitrogen excretion for a man of the order of 10 Gm with that of a rat of the order of 0.1 gram. Expressed in absolute terms, each of these values has significance only in relation to the particular animal from which it was obtained. Units which compare quantities on the per animal basis are biologic units in a sense, but are not broad units which admit comparisons between animals of such widely different size as a rat and a man, nor even between large and small animals of the same species.

Assuming that the basic mechanisms of protein synthesis are the same for all of the mammalian species upon what rational basis can we compare them? It has been demonstrated that energy consumption is approximately proportional to the surface area for a wide variety of mammals.<sup>3</sup> Basal nitrogen excretion in several mammalian species including man has been demonstrated to be proportional to the basal energy requirement.<sup>4</sup> Therefore it appears reasonable to compare the maintenance nitrogen requirements of different animals on the basis of surface area. Such a procedure is not necessarily valid when we are dealing with the construction of new tissue or the reconstruction of depleted tissue. What is the reasonable basis of comparison for feeding in this situation? The ingestion of protein under the conditions of synthesis and storage of new protoplasm falls in a class with many other chemical reactions. The chemist has long expressed additions to synthetic systems in terms of grams, moles, or equivalents of chemical substances per unit of fluid volume or mass. Viewing a kilogram of active protoplasm as such a chemical system, it appears rational to designate additions of protein offered to the system as so many grams per kilogram per day and likewise to compute protein retained and incorporated into the system in similar terms.

Energy intake is another measurement which must be reduced to common terms for comparative purposes. There is an old precedent for this in that basal metabolism has been expressed for many years in terms of calories per square meter of body surface per unit of time. We will not now discuss the significance of this relationship (see Brody<sup>5</sup>), but we have assumed its validity and computed the caloric intakes as calories per square meter of body surface per day (Cal per square meter per day). For computation of the surface area of rats Lee's formula<sup>6</sup> was used, and for the human being, the tables of Dubois.<sup>6</sup>

In the experimental observations, adult male albino rats (Sprague Dawley) weighing initially 288 to 350 grams were depleted of 15 to 25 per cent of their body weight in a period of five weeks on an essentially protein free diet.<sup>7</sup> At the end of the depletion period they were placed in individual metabolism cages. Five diets were constructed offering approximately 0, 1, 2, 3, and 4 Gm of protein per kilogram of body weight per day. The basic composition of the diets was the same as that of the standard repletion diet used in this laboratory.<sup>8</sup> Isodynamic replacement of the carbohydrate by calculated quantities of protein furnished the required protein levels. The protein was a half and half mixture lactalbumin\* and casein†. Fifteen grams of each ration were offered daily to each animal supplying it with 48 calories or between 1,200 and 1,400 Cal per square meter per day. Five animals were used and the diets were rotated from day to day (Table II). The order of rotation for each animal was different. Daily collections of urine, feces, and waste food were made and the rats were weighed. The waste food was weighed

\*Borden's No. 1542

†SMACO vitamin test, General Biochemicals Inc.

and pooled with the urine and feces for Kjeldahl nitrogen analyses. From these determinations the diet analyses and the food intake data the nitrogen balances were computed. Caloric intake was computed from the food intake using the conventional caloric equivalents.

A second experiment was performed using six animals and the 0.1 and 4 Gm per kilogram per day levels of protein intake along with three other diets of varying caloric value and bulk. The procedure used was similar to that described except that the feces were analyzed separately from waste food and urine. Throughout these experiments food waste was minimal averaging 3 per cent and never over 7 per cent of the total offered.

## OBSERVATIONS

A series of observations on the effects of various diets in the repletion of a group of severely starved but otherwise healthy men was published by von Hoesslin in 1919.<sup>2</sup> By comparison with normal height and weight tables<sup>8</sup> we estimated that the subjects had lost between 21 and 29 per cent of their body weight prior to the start of the tests. The repletion diets contained protein from both animal and vegetable sources of which 23 to 58 per cent was animal protein.

TABLE I THE INFLUENCE OF VARYING CALORIC AND PROTEIN INTAKE UPON THE UTILIZATION OF PROTEIN BY MEN SUFFERING FROM SEVERE INANITION. DATA REPRESENT AVERAGES OF FOUR, FIVE OR SEVEN DAY PERIODS ON GROUPS OF FIVE MEN  
(Recalculated from von Hoesslin Arch Hyg 88 147 1919)

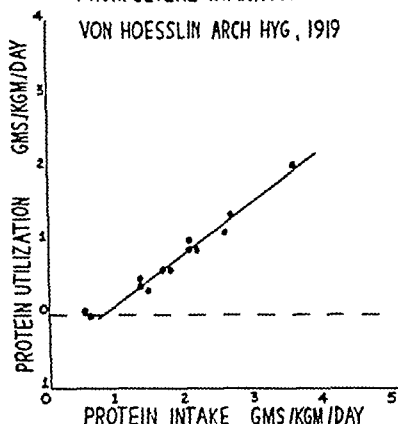
PERIOD	BODY WEIGHT AT START OF PERIOD (KG.)	CALORIC INTAKE (CAL./M <sup>2</sup> /DAY)	NITROGEN INTAKE (GM./KG./DAY)	PROTEIN UTILIZED (GM./KG./DAY)
6	50.0	1400	1.3	0.46
7	49.9	1500	0.6	-0.02
8	49.3	1700	0.5	0.02
1	45.0	1160	1.4	0.32
2	45.1	1700	1.3	0.36
	44.8	1300	2.0	0.88
4	46.5	1440	2.1	0.86
1	48.2	1490	2.0	0.99
2	48.8	1650	2.6	1.34
3	49.0	2730	2.5	1.10
4	52.1	2580	3.5	2.04
5	51.5	1350	1.7	0.61
6	51.5	2650	1.7	0.60

Both caloric and protein intakes were varied and the nitrogen retention under the different conditions was observed. Groups of five men were fed each diet and the period used to test a single diet varied from four to seven days. A summary of the recalculated data is presented in Table I and in Fig. 1 protein utilization is plotted against protein intake. The data in the table demonstrate that at a constant protein intake (1.7 Gm. per kilogram per day) increasing the caloric intake from 1350 to 2650 Cal. per square meter per day had no influence upon protein utilization. On the other hand Fig. 1 demonstrates that protein utilization is proportional to protein intake over the range of protein intake studied.



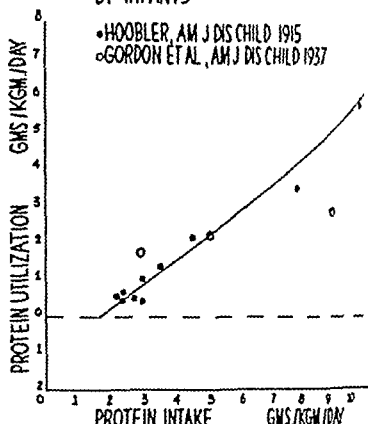
# UTILIZATION OF INGESTED PROTEIN BY MEN SUFFERING FROM SEVERE INANITION

VON HOESSLIN ARCH HYG, 1919



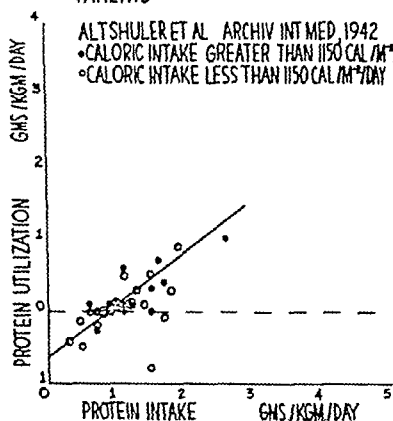
# UTILIZATION OF INGESTED PROTEIN BY INFANTS

• HOOBLER, AM J DIS CHILD 1915  
○ GORDON ET AL, AM J DIS CHILD 1937



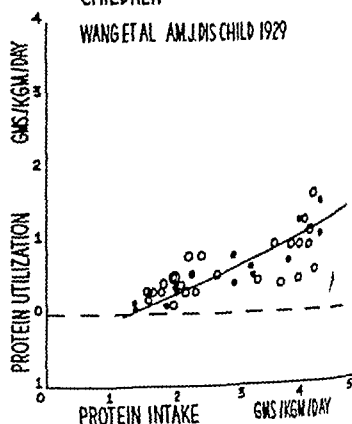
# PROTEIN UTILIZATION BY CANCER PATIENTS

ALTSHULER ET AL ARCHIV INT MED 1942  
• CALORIC INTAKE GREATER THAN 1150 CAL/M<sup>2</sup>/DAY  
○ CALORIC INTAKE LESS THAN 1150 CAL/M<sup>2</sup>/DAY



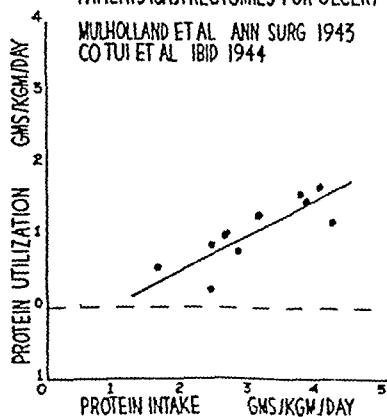
# UTILIZATION OF INGESTED PROTEIN BY NORMAL AND UNDERWEIGHT CHILDREN

WANG ET AL AM J DIS CHILD 1929



# PROTEIN UTILIZATION BY POSTOPERATIVE PATIENTS (GASTRECTOMIES FOR ULCER)

MULHOLLAND ET AL ANN SURG 1943  
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# UTILIZATION OF INGESTED PROTEIN BY NEPHROTIC CHILDREN

FARR AM J MED SCI 1938

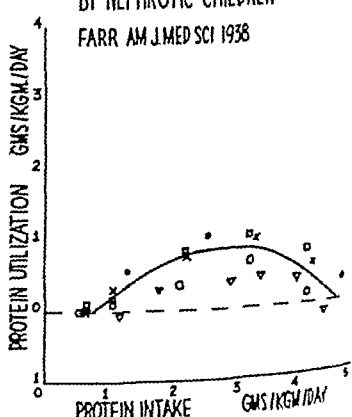


FIG 1—The influence of the level of protein intake on the rate of protein utilization by convalescent adult and growing human beings

Observations were collected on a group of patients suffering from wasting diseases principally cancer. Their usual hospital diet was supplemented with a protein hydrolysate (casein) administered intravenously and nitrogen retention was studied. We have recalculated these data of Altshuler and co-workers<sup>9</sup> for nine cancer patients and plotted them in Fig. 1. The chart demonstrates that protein utilization increases proportionally on the average to the protein intake. Examination of the whole of the observations on the cancer patients by the method of partial correlations brings out the fact that over the entire range of caloric intake used, from 570 to 1,700 Cal per square meter per day, caloric intake had no significant influence upon protein utilization whereas protein utilization had a highly significant correlation with protein intake.

Patients convalescing from gastrectomies for peptic ulcer were fed through orogastric tubes by Mulholland and Co. Tur and colleagues<sup>10, 11</sup>. These patients received protein ranging from 2.4 to 4.2 Gm per kilogram per day and caloric intakes between 1,100 and 2,700 Cal per square meter per day. The protein source was an enzymatic hydrolysate of casein. In Fig. 1 the recalculated data on these patients have been plotted and demonstrate that protein utilization was proportional to the protein intake.

The protein metabolism of a healthy 2-month-old infant was studied by Hoobler<sup>1</sup>. The intake of cows' milk protein was varied from 2.2 to 10.9 Gm per kilogram per day and the caloric intake was maintained between 1,040 and 1,240 Cal per square meter per day with the exception of one period when it rose to 1,570 Cal per square meter per day. The recalculated observations (Fig. 1) demonstrate that this young infant utilized ingested protein at a rate proportional to the protein intake. Cordon and associates<sup>12</sup> studied the protein metabolism of premature infants. Their data (Fig. 1) also demonstrate that the protein was utilized at a rate proportional to the level of protein intake and a little less efficiently at the higher levels of intake than it was utilized by the full-term infant.

Wang and co-workers<sup>14, 15</sup> made extensive studies of the metabolism of normal and undernourished children. Their data show (Fig. 1) that the utilization of ingested protein nitrogen by both groups of children was proportional to the protein intake. The protein was mixed but largely of animal origin. They found that children who were 20 per cent or more below the normal weight standards utilized the protein a little more efficiently than less underweight and normal children<sup>15</sup>. The absolute proportion of ingested protein retained by Wang's subjects averaged substantially less than that retained by the normal infant or the starved adults. Metabolism of nephrotic children at protein intake levels varying between 0.6 and 4.8 Gm per kilogram per day was studied by Farr<sup>16</sup>. His data (Fig. 1) indicate that even these children utilized increasing quantities of protein as the protein intake was increased up to about 3 Gm per kilogram per day but beyond this as the intake was further increased the utilization fell sharply.

In Table II are the data derived from the experiments with rats and in Fig. 2 the rate of protein utilization is plotted against the rate of protein intake. It is obvious that over the range of intake studied the rate of utilization of a

TABLE II PROTEIN UTILIZATION BY PROTEIN DEPLETED RATS ON ADEQUATE CALORIC INTAKES

ANIMAL NUMBER	PERIOD NUMBER	BODY WEIGHT (GM)	CALORIC INTAKE (CAL/M <sup>2</sup> /DAY)	PROTEIN INTAKE (GM/KG/DAY)	PROTEIN UTILIZED (GM/KG/DAY)
<i>Experiment 1</i>					
1	1	285	1260	0.2	-0.8
	2	284	1240	1.1	-0.1
	3	288	1190	2.1	0.0
	4	286	1230	3.7	2.5
	5	290	1210	4.5	3.2
2	1	290	1230	3.9	2.0
	2	286	1200	0.2	-1.1
	3	292	1230	1.2	-0.2
	4	290	1250	2.6	0.6
	5	296	1220	2.6	1.1
3	1	282	1270	2.9	1.1
	2	280	1260	3.9	2.6
	3	282	1250	0.2	-1.0
	4	285	1260	1.4	0.0
	5	288	1250	2.5	1.1
4	1	236	1400	2.0	0.8
	2	234	1410	3.0	1.8
	3	238	1410	4.1	2.9
	4	238	1380	0.3	-0.5
	5	240	1370	1.4	-0.2
5	1	251	1380	1.1	-0.4
	2	248	1380	2.0	0.8
	3	252	1370	3.0	1.6
	4	251	1370	4.7	2.8
	5	256	1350	0.3	-0.6
<i>Experiment 2</i>					
6	2	254	1260	4.7	3.0
	5	276	1200	1.7	0.5
	6	253	1350	0.2	-0.9
7	1	258	1360	0.2	-1.3
	3	251	1270	4.2	2.7
	6	252	1270	1.9	0.5
8	1	259	1250	1.9	0.4
	2	259	1330	0.2	-1.5
	4	252	1270	4.8	2.8
9	2	256	1260	1.9	0.7
	3	256	1370	0.2	-0.9
	5	284	1180	4.8	2.7
10	1	258	1270	1.9	0.2
	4	255	1340	0.2	-1.4
	6	264	1260	4.6	2.7
11	1	262	1260	4.6	2.7
	4	251	1300	1.9	0.2
	5	286	1260	0.2	-1.0

high quality protein is proportional to the level of protein intake, providing the caloric intake is adequate. Comparison of these data with those of Experiment 2 in the preceding publication<sup>1</sup> shows that the two follow the same general pattern. Utilization of protein in the present experiments appears to be at a somewhat higher level than in the preceding one. This may be due in part to the fact that the periods of observation here were shorter, and that therefore the avidity of the tissues for protein remained closer to maximal whereas with the fourteen day experiments the growth potential was already significantly decreased toward the end of the period of observation.

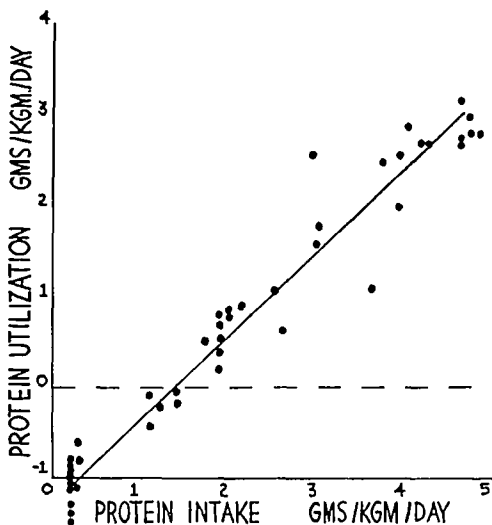


Fig. 2.—Utilization of ingested protein by protein depleted rats on an adequate caloric intake at varying levels of protein intake

#### DISCUSSION

The foregoing observations demonstrate that the protein depleted and the growing animal, man or rat given an adequate caloric intake utilize protein at a rate which is proportional to the rate of protein intake. For man a similar pattern of reaction is apparent not only in convalescence from starvation alone but also in convalescence from certain debilitating diseases. The similarity in the performance of the depleted rats and the starved men is strikingly evident when one compares Fig. 2 with Fig. 1.

At first glance the similarity in response to protein ingestion of these two species of mammals of such different size and external characteristics seems remarkable. These facts when compared with some of the many other observations on mammals appear less startling. They become not unique but part of a great body of evidence accumulated over many years which points to the conclusion that the basic mechanisms underlying the organization and functioning of the tissues of the different species of animals are fundamentally similar. Let us examine a little of the evidence from the biochemical standpoint having in mind principally, a comparison of the rat and the human being. Body temperatures of rats and men are almost identical and their basal metabolic rates do not differ greatly. In broader terms the basal energy requirements of animals varying in size from the mouse to the elephant have been found to be proportional to a function of the body mass which approximates mathematically

the surface area.<sup>3</sup> The basal nitrogen excretion is related to the basal metabolism, thus rat, rabbit, guinea pig, man, and swine have been shown to excrete in urine an almost identical quantity of nitrogen per basal calorie per day.<sup>4</sup> The same amino acids, with the possible exception of histidine,<sup>17</sup> appear to be indispensable for the growth and maintenance of the rat and of man.<sup>18, 19</sup> Moreover we have evidence which suggests that the essential amino acids are needed in similar relative proportions in the two species for maintenance and for growth.<sup>18</sup> Viewing the matter broadly, there is a general quantitative, as well as qualitative, pattern into which rats and men as well as other mammals fit.

Von Noorden acknowledged the broad problem of the relationship of the level of protein intake and the caloric intake to the rate of repletion of lost tissue.<sup>20</sup> He outlined the types of experiments needed to settle the question, but presented evidence from only a single type of experiment, namely, that in which approximately the same quantity of protein per kilogram of body weight was fed per day to patients convalescing from debilitating infections while the caloric intake was varied. He concluded from these observations that high caloric intakes were more favorable than high protein intakes in inducing more rapid rates of rehabilitation.

Recently Keys has emphasized the efficacy of high caloric intakes to further rehabilitation in starved men.<sup>21</sup> He states that, "In relief feeding, calories are of overwhelming importance. Within reasonable limits every increase in calories is associated with an increased rate of recovery." He states further that, "Extra vitamins and proteins had very little apparent effect on the rate or course of recovery." The evidence which we have presented is not in accord with these statements, and demonstrates that while caloric intake is a factor, it is not the only factor. Moreover, with an adequate caloric intake, the level of protein intake and the quality of the protein become the limiting factors.

The role of caloric intake in problems of nutrition has been vastly overdrawn for as Cathcart<sup>22</sup> has stated, "We do not live on calories." Caloric value is simply a very convenient physical standard for the assessment of diets but merely because such a standard has proved of great utilitarian value there is no real justification for placing this standard as the foundation stone of hypotheses framed to offer an explanation of cellular activity. Many writers are obsessed with the idea of the calorie, forgetting that the organism is certainly not a heat engine. It is perfectly true that calories are a measure of heat, but it must not be forgotten that we do not consume actual heat units but only potential heat-giving substances which can eventually be degraded to the form of heat and be measured as such. The thermal aspect of nutrition is unduly stressed, for, while heat may be a necessary product of tissue activity, it is after all a by-product."

The aim when feeding sick, convalescent, starved, and growing individuals is not the mere maintenance of nitrogen equilibrium but the storage of significant quantities of protein as new tissue. The preceding observations demonstrate clearly that certain describable conditions must be satisfied to attain this goal. The conditions are as follows: (1) caloric intake must be adequate to cover needs for maintenance, physical exertion, tissue synthesis storage and

waste, (2) a protein of high biologic value must be fed, (3) the protein must be fed in excess of the quantity needed to maintain nitrogenous equilibrium and in general the higher the level of protein intake the greater the rate of synthesis, (4) other dietary essentials (vitamins minerals and so on) must be adequate

With these general propositions before us we are in a position to make more specific estimates of caloric and protein intakes for convalescence. But first we must clarify one further point. It has been customary to compute diets on the basis of total caloric content and protein content. The total caloric content has practically always included the caloric equivalent of the protein. In convalescence from starvation of any origin the maximal utilization of protein for tissue reconstruction is desired. Therefore it appears rational to include protein in the computations both as a source of amino acids for tissue fabrication and as a source of fuel for energy consuming reactions. The rational procedure is to feed protein as a source of building material and compute the energy value of the diet independent of its protein content on the basis of its carbohydrate and fat content.

An adequate caloric intake for a convalescent man can be estimated from considerations of the known basal requirements requirements for physical exertion, protein synthesis and waste. For an average sized man (surface area 1.7 square meters) afebrile and at bed rest the estimate is 2,600 calories per day or 1,500 Cal per square meter per day\*. Such an allowance represents not the absolute minimum but is comparable to the standard ration used by us in many animal experiments which allows approximately 1,460 Cal per square meter per day of nonprotein energy.

The animal data<sup>1</sup> indicate that a protein intake level of 10 Gm per kilogram per day or more would provide better rates of protein synthesis than 2 or 4 Gm per kilogram per day. Unfortunately it is not possible to feed adult human beings at this level. The reason for this becomes clear if one compares the per kilogram energy consumption of rats and men. The relatively large surface to mass ratio of the rat allows a 10 Gm per kilogram per day protein intake to represent only a fraction of the total caloric intake (about one eighth) whereas this level of protein intake would represent almost the entire daily caloric needs of a man. But the data indicate that a man can easily use up to 4 Gm per kilogram per day. Protein intakes of the order of only 1 Gm per kilogram per day will be ineffective in inducing significant degrees of protein storage. While this level of protein intake is the commonly accepted standard for maintenance of nitrogen equilibrium in a healthy individual it is inadequate for the depleted

The estimate was arrived at in the following manner. Basal requirements of a normal man are approximately 1,000 Cal per square meter per day and a starved man needs even less. For the activity associated even with bed rest 30 per cent of the basal requirements were added. From thermodynamic considerations it has been shown<sup>2</sup> that the peptide bond contains a free energy equivalent of 7.5 calories per mole. Assuming only a 10 per cent efficiency in the biologic synthesis it would take 83 calories to synthesize 100 Gm of protein. To allow for the synthesis of 100 Gm of protein with some excess .00 calorie were added for this factor. Excluding waste energy the needs for the described situation are  $1.7 \times 1.00 + .00 = 2.410$  calories. According to Rubner<sup>3</sup> about 8 per cent of the energy intake is lost in the feces therefore 190 calories were added for this factor totalling 2,600 calories.

individual in whom nitrogen must be stored. We cannot overemphasize the fact that there is a fundamental difference between the maintenance of the equilibrium state in the fully grown and healthy adult and the construction of new tissue in the depleted or growing individual. In order to obtain good rates of tissue synthesis under these latter conditions it is necessary to feed at protein intake levels of the order of 2 to 4 Gm. per kilogram per day. An examination of the data (Fig. 1) demonstrates that the higher protein intake levels have two advantages: first, a greater absolute rate of protein storage and second a higher gross efficiency of protein utilization.

#### SUMMARY AND CONCLUSIONS

For the purpose of evaluating the respective roles of the level of caloric intake and the level of protein intake in the restoration of protein stores of depleted animals, we have presented further experiments on rats and a re-evaluation of observations from the literature on man. From these data the following conclusions are drawn: (1) In both groups of mammals with protein intakes ranging from 0.5 to 4 Gm. per kilogram of body weight per day the utilization of protein for tissue synthesis is proportional to the protein intake. (2) The rate of protein utilization is independent of the caloric intake at caloric intake levels above those which supply the needs for maintenance, synthesis, storage, and waste. The estimated nonprotein energy needs for an adult male man at bed rest in the absence of other metabolic stimulants is approximately 1,500 calories per square meter of body surface per day. (3) Since the therapeutic goal is not the mere maintenance of nitrogen equilibrium but the fabrication of significant quantities of body protein, protein intake levels of 2 to 4 Gm. per kilogram per day are demanded for the rapid rehabilitation of a depleted person. At these levels there is both a higher gross efficiency of protein utilization and a greater absolute rate of protein gain.

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# THE DETERMINATION OF THE NITROGEN BALANCE INDEX OF A NEW LYOPHILIZED AMINO ACID PREPARATION IN PROTEIN-DEFICIENT PATIENTS

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PROTEIN products and protein hydrolysates intended for human nutrition have their principal usefulness in the repletion of protein deficient patients. The criterion for clinical acceptability of such a product has been its ability to produce positive nitrogen balance in protein deficient subjects. The quantity required for positive balance must be of the same order as that of the mixture of proteins in the common foods. If a much greater quantity is required it is recognized that the biologic value of the investigated product is relatively low, at least under the conditions of administration utilized.

Yet it has been increasingly clear from nitrogen balance experiments that human subjects with protein deficiency differ greatly from each other in the nitrogen intake of a particular protein required for nitrogen equilibrium, even when such subjects are chosen from an apparently homogeneous group. For example, in a nitrogen balance study of a casein hydrolysate in this laboratory some subjects came into positive balance with a daily intake of 60 Gm. of amino acids, others with 90 Gm. and still others only after 120 grams. An occasional patient could not be brought into balance with as much as 135 Gm. of amino acids equivalent to 16.9 Gm. of protein nitrogen. Comparable findings were obtained in the study of an oral lactalbumin derivative.<sup>1</sup> The calculation of the *average* requirement of a particular protein in experiments such as these is justifiable only if the number of experiments is large and if the deviations from the average are not too great. The utilization of such averages for comparison of one product with another is obviously fraught with danger.

Recent feeding experiments with protein depleted dogs by Allison and co-workers<sup>2, 3</sup> have demonstrated that even laboratory animals under careful supervision show wide variations in protein requirements for nitrogen equilibrium. These investigators have been able to show that the nitrogen intake at equilibrium depended upon the nitrogen excretion on a protein free diet, the latter excretion being a measure of the so called endogenous protein metabolism. The lower the nitrogen excretion ( $NE_0$ ) on the protein free diet the lower was the requirement for equilibrium. If the nitrogen balance (NB) obtained on successive nitrogen intakes, the latter being calculated as absorbed nitrogen (AN), was plotted against the absorbed nitrogen, a curve was obtained which in the region of negative and low positive nitrogen balance was a straight line.

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The equation for this relationship was  $NB = K (AN) - NE_0$ . The value  $K$  was the slope of the line and it was found to be a measure of the biologic value of the protein used in the study. It tended to be a constant for any given protein even when the nitrogen intake at equilibrium varied considerably. Thus the calculation of  $K$  appeared to be a much better characterization of the biologic value of the protein than the nitrogen requirement at nitrogen equilibrium. The values for  $K$  were usually less than 1; the nearer to 1 they were the greater the fraction of absorbed nitrogen that was retained. Casein for example was found to have a  $K$  value of about 0.80. Allison has called  $K$  the nitrogen balance index.

It appeared to us during the course of a study of the clinical acceptability of a new casein hydrolysate prepared for parenteral use, that the methods and equation of Allison might be applicable to nitrogen balance studies in human subjects. The present investigation was therefore undertaken to find out whether the nitrogen balance index of this amino acid preparation tended to be constant in the nitrogen balance experiments with twenty patients with chronic protein deficiency and whether such an index was a better criterion of biologic value than the nitrogen intake at nitrogen equilibrium.

#### METHODS

The study was carried out in a metabolic unit staffed twenty-four hours a day by nurses specifically chosen for this function. The urine specimens were collected for each twenty-four hour period in closed bottles containing toluene and were sent each day to the laboratory for analysis. The subjects were under constant supervision to avoid the loss of specimens. A further check was the determination of the daily creatinine excretion.

Since none of the twenty patients chosen had any difficulty taking oral alimentation this avenue was utilized as a means of furnishing an adequate intake of calories from carbohydrate and fat. The oral dietary was designed to be virtually free of protein so that the only significant source of nitrogen would be the parenterally injected amino acids. However, the food orally ingested could not be made rigidly free of nitrogen as might be possible with laboratory animals or volunteer human subjects. The dietary consisted of a carbohydrate drink\*, tapioca and cornstarch puddings, orange juice, tomato juice and a puréed leafy vegetable. Since the amount of nitrogen in the foods chosen was low (between 350 and 500 mg) and since it was derived from proteins of poor absorbability and poor biologic value, the error involved in the inclusion of this nitrogen source was probably of no consequence. It was probably no higher than the nitrogen lost in perspiration, even though the studies were carried out in cool weather and was therefore not included in the calculation. The oral carbohydrate and fat plus the intravenously injected dextrose furnished approximately 2,000 calories per day, but slight variation above or below this quantity could not be avoided.

The amino acid preparation under study was a casein acid hydrolysate fortified with tryptophane and prepared as a lyophilized solid in 8x10 cc flasks†. The nitrogen content and the percentage composition of essential amino acids in the preparation are shown in Table I. A substantial portion of the glutamic and aspartic acids present in the original hydrolysate had been removed by a special process. Each flask contained 60 c.c. of the lyophilized solid which could be reconstituted to a perfectly clear solution with water, physiologic saline or dextrose saline solutions. The solution could be made up in 5

Ind. The carbohydrate used was Carbo-Cee furnished by H. W. Kinney and Sons, Columbus.

†The product called Amino Acids—I C Lyophilized was furnished by The Flockmeyer Division, Interchemical Corporation, Union, N. J.

7.5, or 10 per cent concentrations of the amino acids. Most of the injections were made with a 5 per cent solution at a rate between 50 and 60 drops per minute in order to make sure that there would be no vomiting. But even when the 10 per cent solution was utilized in these and other experiments, if this rate was not exceeded nausea or vomiting seldom occurred. Repeated injection in the same vein tended to produce thrombophlebitis, as has been found with other protein hydrolysate preparations used. But this circumstance could be mitigated by frequent change of the veins utilized and by starting with the most peripheral veins and proceeding cephalad. The needles used were No. 19 gauge and were introduced with a minimum of trauma to the veins.

TABLE I CHEMICAL ANALYSIS OF AMINO ACID PREPARATION USED IN THIS STUDY  
(AMINO ACIDS, I.C., LYOPHILIZED)

	(%)
Nitrogen	
Total	13.4
Alpha amino	10.0
% Alpha amino nitrogen of total nitrogen	75.0
Essential amino acids (by microbiologic assay)	
Arginine	3.8
Histidine	2.7
Isoleucine	7.4
Leucine	10.6
Lysine	8.5
Methionine	3.0
Phenylalanine	5.5
Threonine	5.0
Tryptophane	0.5*
Valine	7.3
Moisture	12.0
Ash	0.9

\*One per cent DL-tryptophane added

All patients were first placed on the protein free diet for a period of three days or more to establish the endogenous nitrogen excretion level. Then daily infusions of amino acids were given in addition to the oral alimentation in quantities known not to produce positive balance. After three or four days of this regimen, the quantity of amino acids was further increased and maintained at the new level for three or more days. With successive increases a nitrogen balance was achieved that was barely positive. Further increases were then made at a more rapid rate to determine, if possible, the maximal level of utilization.

Careful attention was paid to the vitamin requirements of the patients. Ascorbic acid was given in doses of 500 mg per day, thiamin and other members of the vitamin B complex were given as Betulin Complex,\* one cc of which provided 5 mg thiamin chloride, 2 mg riboflavin, 75 mg nicotinamide, 25 mg calcium pantothenate, and 5 mg pyridoxine hydrochloride.

Quantitative analyses of the nitrogen content of blood, urine, feces, and foods as well as that of the hydrolysate were carried out by the photometric macro Kjeldahl method described by Hoffman and Osgood.<sup>5</sup> They were frequently checked by macro Kjeldahl determinations involving distillation and titration. For stool analysis, the preparations were first homogenized in water by the addition of concentrated sulfuric acid, as suggested by Peters and Van Slyke.<sup>6</sup> Plasma volumes were estimated from a single ten minute plasma sample after the injection of 10 mg of Evans blue as described by Greger.<sup>7</sup> A calibration curve was made in each analysis with the control serum. To avoid variations in the fat content of the control and dye containing samples, the tests were made at least twelve hours after the previous meal or infusion of amino acids. Hematocrit determinations were made on heparinized blood by the method of Wintrobe and Landisberg.<sup>8</sup> Urine and stool analyses were made daily, blood analyses including plasma volume estimations were made usually at weekly intervals, at which time the patient was weighed.

\*Furnished by Eli Lilly & Company, Indianapolis, Ind.

TABLE II NITROGEN BALANCE STUDIES IN TWENTY PROTEIN DEFICIENT PATIENTS  
DETERMINATION OF MINIMAL AMINO ACID INTAKE PRODUCING NITROGEN BALANCE

PATIENT	AGE (YR)	DIAGNOSIS	WEIGHT (KG)	AMINO ACID INTAKE (GM/DAY)	N INTAKE		N EXCRETION (GM/DAY)	N BALANCE		N REQUIREMENT FOR EQUILIBRIUM (INTERPOLATED) (MG/DAY)	K (FROM FIG 1)
					(GM/DAY)	(MG/DAY)		(GM/DAY)	(MG/DAY)		
J V 1	61	Cancer of esophagus	45.9	0	0	0	1.8	-1.8	-40	50	0.97
				26	3.4	7.1	2.0	+1.4	+30		
				32	6.7	14.6	2.2	+4.5	+98		
				82	10.8	27.1	8.0	+2.8	+61		
S D 2	62	Gastric ulcer	55.4	0	0	0	4.0	-4.0	-72	158	0.46
				29	3.7	6.7	5.7	-2.0	-36		
				38	7.4	13.4	8.1	-0.7	-13		
				87	11.1	20.1	10.0	+1.1	+20		
				116	14.8	26.8	14.2	+0.6	+11		
F I 3	48	Cancer of esophagus	62.0	0	0	0	5.4	-5.4	-87	116	0.74
				25	3.5	5.3	7.9	-4.6	-74		
				50	6.6	10.6	5.1	+1.5	+24		
				70	9.9	15.0	6.7	+2.6	+42		
				82.5	10.7	17.3	8.1	+2.6	+42		
T Z 4	65	Cancer of esophagus	41.6	0	0	0	7.8	-7.8	-188	340	0.54
				29	4.1	9.9	6.5	-2.4	-58		
				70.5	8.8	21.2	9.9	-1.1	-26		
				94.0	11.0	26.4	12.8	-1.8	-4		
				150.0	19.8	47.6	17.3	+2.5	+60		
H A 5	59	Malnutrition aplastic anemia	47.8	0	0	0	5.3	-5.3	-111	185	0.61
				26.5	3.4	7.1	6.6	-3.2	-67		
				53.0	6.8	14.2	7.5	-0.7	-15		
				79.5	10.2	21.3	9.3	+0.9	+19		
				106.0	13.6	28.4	10.7	+2.9	+61		
R S 6	39	Malnutrition	52.7	0	0	0	4.3	-4.3	-82	150	0.49
				32.9	4.1	7.8	5.8	-1.7	-32		
				70.5	8.8	16.7	9.0	-0.2	-4		
				94.0	11.0	20.9	9.9	+1.1	+21		
				150.0	19.8	37.6	13.2	+6.6	+125		
B O 7	65	Cancer of esophagus	58.9	0	0	0	9.9	-9.9	-168	—	—
				50	6.6	11.2	12.4	-5.8	-98		
				55	3.3	5.6	7.6	-4.3	-73		
				50	6.6	11.2	8.9	-2.3	-39		
				70	9.4	15.2	16.2	-6.8	-115		
M C 8	45	Splenemia	43.5	0	0	0	6.8	-6.8	-156	155	1.02
				29.2	3.7	8.4	5.9	-2.1	-48		
				38.4	3.3	16.8	6.8	+0.5	+11		
				87.6	11.0	25.1	9.8	+1.2	+28		
				116.8	14.6	33.5	13.5	+1.1	+25		
I H 9	45	Cirrhosis with ascites	37.9	0	0	0	1.5	-1.5	-26	42	0.52
				26	3.3	5.7	3.1	+0.2	+7		
				52	6.7	11.5	3.2	+3.5	+60		
				104	13.4	31.1	6.8	+6.8	+117		
				104	13.4	31.1	6.8	+6.8	+117		
A B 10	56	Chronic ulcerative colitis nontoxic goiter	39.6	0	0	0	3.5	-3.5	-88	10	0.44
				28.5	3.6	9.9	4.7	-1.1	-28		
				57.0	7.2	18.2	7.9	-0.7	-18		
				85.5	10.8	27.3	9.5	+1.3	+33		
				113.0	14.3	36.4	11.0	+2.4	+61		

(Continued on following page)

TABLE II—CONT'D

PATIENT	AGE (YR)	DIAGNOSIS	WEIGHT (KG)	AMINO ACID IN TAKE (GM/DAY)	N INTAKE		N EXCRETION (GM/DAY)	N BALANCE		N REQUIREMENT FOR EQUILIBRIUM (INTERPOLATED) (MG/DAY)	K (FROM FIG 1)
					(GM/DAY)	(MG/KG/DAY)		(GM/DAY)	(MG/KG/DAY)		
M M 11	66	Leg ulcers	59.9	0 30 44 60 70 90 174	0 3.8 5.5 7.7 8.8 11.5 22.1	0 64 92 128 148 192 369	6.4 7.0 6.6 9.7 8.9 8.9 17.8	-6.4 -3.2 -1.1 -2.0 -0.1 +2.6 +4.3	-107 -53 -18 -33 -2 +43 +72	140	0.79
L M 12	60	Huge thigh ulcer	41.5	0 27.5 44.0 55.0 71.5 95.0	0 3.5 5.5 7.1 9.0 12.1	0 84 133 171 217 292	5.2 5.6 4.9 6.3 9.0 11.2	-5.2 -2.1 +0.6 +0.8 0 +0.9	-125 -51 +18 +19 0 +22	146	0.84
P M 13	61	Wound separation, bowel obstruction	53.2	0 28 45 56 84 112	0 3.6 5.7 7.2 10.8 14.3	0 68 107 136 204 269	5.4 5.2 6.6 6.1 8.3 12.3	-5.4 -1.6 -0.9 +1.1 +2.5 +2.1	-101 -30 -17 +21 +47 +39	140	0.71
A T 14	52	Infected mastectomy wound	39.8	0 27.5 40.0 50.0 75.0 108.0	0 3.5 5.0 6.3 9.5 13.4	0 88 126 158 239 340	3.0 3.2 5.3 5.4 7.0 10.4	-3.0 +0.3 -0.3 +0.9 +2.5 +3.0	-75 +8 -8 +23 +63 +75	125	0.58
J W 15	68	Cancer of rectum	43.2	0 30 40 50 59 118	0 4.0 5.3 6.6 7.9 15.8	0 92 123 153 183 366	2.4 4.3 5.7 6.0 7.6 11.8	-2.4 -0.3 -0.4 +0.6 +0.3 +4.0	-56 -7 -9 +14 +7 +93	115	0.49
I T 16	72	Leg ulcer	55.2	0 30 47 59 118 180	0 4.0 6.5 8.1 16.2 23.9	0 73 117 146 292 433	4.7 4.4 5.5 5.6 11.9 13.8	-4.7 -0.4 +1.0 +2.5 +4.3 +10.1	-85 -7 +18 +45 +78 +183	100	0.95
M T 17	65	Femoral hernia	50.0	0 20 56 62 110	0 2.7 7.6 8.5 14.6	0 54 152 170 290	1.5 2.5 4.8 4.8 12.2	-1.5 +0.2 +2.8 +3.7 +2.4	-30 -4 +56 +74 +48	48	0.63
V E 18	48	Essential hypertension	39.4	0 30 43 62 110	0 4.0 5.9 8.5 14.6	0 102 150 216 371	2.6 3.4 4.2 6.1 12.1	-2.6 +0.6 +1.7 +2.4 +2.5	-66 +15 +43 +61 +63	90	0.71
T O 19	47	Gastrojejuno colic fistula	47.3	0 34 57 114 171	0 4.5 7.5 15.0 22.5	0 95 159 318 477	2.9 2.7 6.6 8.1 15.1	-2.9 +1.8 +0.9 +6.9 +7.4	-61 +38 +19 +146 +156	98	0.62
E R 20	58	Bronchiectasis	54.5	0 28 55	0 3.7 7.3	0 68 134	2.4 3.8 4.2	-2.4 -0.1 +3.1	-44 -2 +31	62	0.70
Average and standard deviations										129 ± 66 (51%)	0.68 ± 16 (24%)

## RESULTS

*Minimal Requirements for Positive Nitrogen Balance*—Positive nitrogen balance was achieved in nineteen of the twenty subjects studied. The data are shown in Table II. Patient 7, who had a carcinoma of the esophagus had not come into positive nitrogen balance on an intake of 182 mg nitrogen per kilogram per day. This patient might possibly have achieved positive balance at a higher level of intake, but he could not be kept on the study any longer. The remaining nineteen patients showed a wide variation in the intake needed for positive balance, ranging from 57 to 476 mg nitrogen per kilogram daily. But these values had little meaning since the levels of positive balance achieved with these quantities varied considerably. Much more significant were the nitrogen intakes required for each nitrogen equilibrium. These quantities could

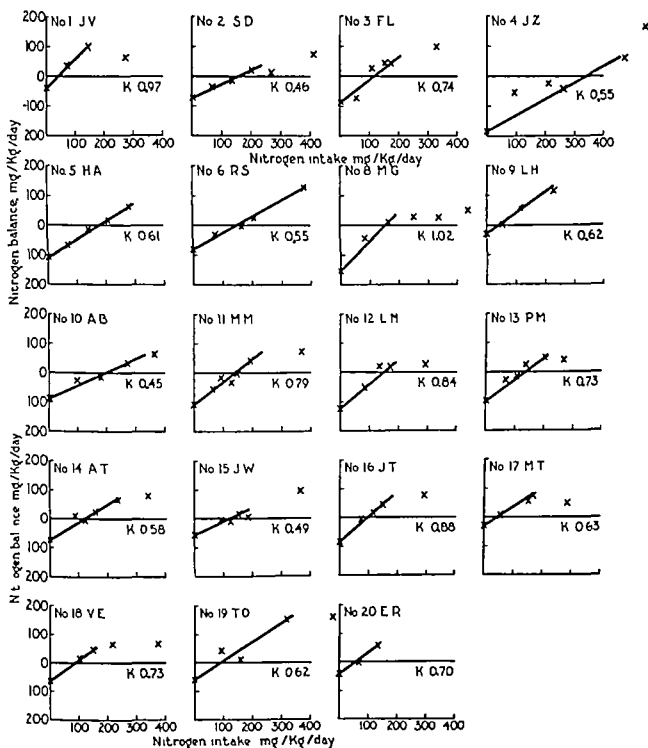


Fig 1—Nitrogen balance curves in nineteen subjects in whom positive nitrogen balance was achieved

be estimated only by interpolation from the curves obtained by plotting nitrogen balance against nitrogen intake (Fig 1) Of the nineteen curves, thirteen were reasonably good straight lines in the region of negative and low positive balance, the remaining six required some arbitrariness for the establishment of straight lines. The range of nitrogen intake levels required for nitrogen equilibrium in all nineteen cases was 42 to 340 mg per kilogram per day. The arithmetic mean was 129 mg and the standard deviation, 66 mg, which was 51 per cent of the mean. If only the thirteen good cases were chosen, the range was 42 to 197 mg per kilogram per day, with a mean of 122 mg and a standard deviation of 48 mg, or 39 per cent of the mean. Thus it was evident that though the range of nitrogen intakes required for nitrogen equilibrium was narrowed if the proper experiments were chosen, that range was still broad.

*Nitrogen Balance Index*—As has been stated, thirteen of the nineteen nitrogen balance curves were straight lines in the region of negative and low positive nitrogen balance. Even the remaining six curves did not show great deviations from a straight line, but their exact slopes were difficult to determine

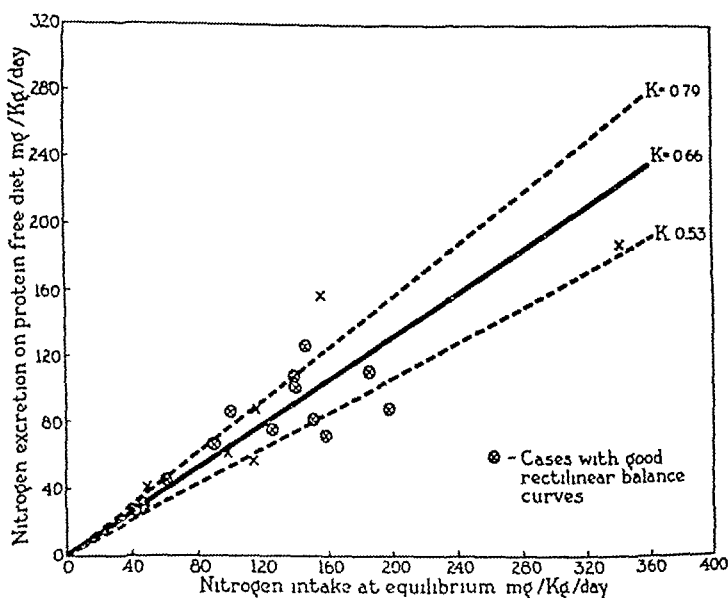


Fig 2—Relation of nitrogen intake at nitrogen equilibrium to nitrogen excretion on a protein-free diet ( $K$  is the ratio of nitrogen excretion on a protein free diet to the nitrogen intake at nitrogen equilibrium)

These straight-line curves obeyed the equation shown earlier. (Nitrogen intake in these experiments was equal to absorbed nitrogen if the small amount of ingested nitrogen which was absorbed from the intestine was ignored.) Thus these experiments confirm in human subjects with protein deficiency produced by disease the findings of Allison and Anderson<sup>3</sup> for dogs with induced protein deficiency.

The  $K$  values for the equations of the curves shown in Fig 1, which were the slopes of these curves and which were most easily calculated from the ratio of nitrogen excretion on a protein-free diet to the nitrogen intake at nitrogen

equilibrium, ranged from 0.45 to 1.02. The mean was 0.68 and the standard deviation was 0.16, or 24 per cent of the mean. In the thirteen cases with good straight line curves  $K$  ranged from 0.45 to 0.88 and the mean was 0.66 with a standard deviation of 0.13 or 20 per cent of the mean. In consideration of the difficulties of nitrogen balance studies in such patients as well as the many potential sources of error in the nitrogen analyses the  $K$  values arrived at in these experiments showed remarkably small deviation from the average. These findings also offered corroboration of Allison's conclusion that the  $K$  values tended to be constant for any particular protein. This approximate constancy is graphically illustrated in Fig. 2 where nitrogen excretion on the protein free diet has been plotted against the nitrogen intake at equilibrium. The scatter points are closely bunched along a straight line which has the slope of mean  $K$  value 0.66. The dotted lines demarcate the standard deviation of the  $K$  values. Both the group of thirteen good experiments and the six more doubtful ones fall equilly well into the wedge within the boundaries of this standard deviation.

Fig. 2 also brings out the important corollary to Allison's equation that there was no tendency to constancy of nitrogen intake at equilibrium but that that value depended upon the endogenous nitrogen metabolism as represented by the nitrogen excretion on a protein free diet. The higher the latter the higher was the nitrogen intake required to produce equilibrium. In this group of patients four showed remarkably low nitrogen excretions in the control protein free periods (Patients 1, 9, 17 and 20). At first these low excretions were blamed on losses of specimens but careful examination failed to reveal any such technical carelessness. Besides the urine volumes in these cases were large the specific gravities low and the creatinine excretions generally constant. Furthermore, these four subjects showed  $K$  values very close to the average and the nitrogen balance curves were good approximations of straight lines. The low excretions were therefore probably genuine. The nitrogen intakes at equilibrium for these four patients were at unusually low levels of 42 to 62 mg per kilogram per day. On the other hand those patients with higher endogenous nitrogen excretions all showed higher intakes at equilibrium. None of this group had the unusually high excretions found by Co Tui and co workers<sup>9</sup> in patients with burns or other conditions producing the so called catabolic assault. If there were such patients they likely would have shown much higher nitrogen intakes at equilibrium than seen in Fig. 1 if the  $K$  values were of the same order as the values found here.

The unusually high  $K$  value of 1.02 might represent a technical error especially in the determination of the endogenous excretion. An occasional subject when placed on the protein free high carbohydrate high fluid diet excreted relatively large quantities of nitrogen for several days before reaching a stable excretion. Thus with only a three or four day control period the patient might still not show the true endogenous excretion. If such a point was used as the pivotal point for drawing the straight line curve the slope would tend to be too steep making  $K$  too high. If however, this value was not due to error it



meant that in that particular patient the addition of protein to the diet spared endogenous metabolism. This phenomenon has been suggested by Allison in some of his balance studies with dogs.

*Maximal Tolerance of the Hydrolysate*—Of the nineteen subjects carried to the point of good positive balance, all but two finally received more than 100 Gm of the protein hydrolysate daily for at least three days. As much as 147 Gm were given daily to eight patients and 200 Gm to two patients for three days. No effort was made in these experiments to extend the injections beyond this period, and it is likely that some extension could have been made before the development of findings of intolerance such as nausea, vomiting, anorexia, or progressive thrombophlebitis. It may be significant that the greatest weight gains occurred in the patients who received as high as 200 Gm of amino acids daily. When the 10 per cent solution was used, the injection of 60 Gm usually required two to three hours for administration. Thus 180 Gm could be injected if need be, in six to nine hours and there would still be time for additional saline or glucose injection without infringement on the period of sleep.

The nitrogen balance curves in the region of high intake were not so steep as those in the region of negative or low positive balance. In fact, in several cases the curves became horizontal, indicating a ceiling of nitrogen retention. In one subject, however, a positive nitrogen balance of 13 Gm was achieved and in another, 10 grams. In the remaining patients the positive nitrogen balance increased only slowly with higher intakes, which posed the problem of whether the increased quantities injected produced enough additional retention of nitrogen to warrant the effort.

It has been maintained<sup>10</sup> that parenterally injected amino acids so reduce the appetite as to cause a diminution of the oral intake of food. This phenomenon occurred in only six of the twenty patients studied here, as demonstrated by a slight drop in the caloric consumption as the quantity of amino acids injected was increased. Three of these patients were quite ill. In six other patients the total caloric intake actually increased slightly, that is, the patients requested and received a greater quantity of the high-carbohydrate drink. In the remaining eight patients the caloric intake remained constant throughout the study. In all cases the carbohydrate intake was nearly constant enough so as not to have any influence on the nitrogen requirement. There was therefore no confirmation of the idea that the injection of amino acids had a deleterious effect upon the over-all appetite of the patient, even though immediately after an injection there was a temporary sense of fullness.

*Effect Upon Circulating Proteins*—The period of positive nitrogen balance was not extensive enough in these experiments to produce the expectation of a great gain in circulating proteins. The data shown in Table III indicate that though nearly all patients had marked hypoproteinemia there was little change for the better in the quantity of circulating proteins during the study. In fact in nine subjects there was a significant fall in the quantity of circulating proteins. In the five instances of rise of circulating proteins this rise was associated with an increase in plasma volume rather than an increase in serum protein concentration.

TABLE III BLOOD PROTEIN STUDIES IN TWENTY PROTEIN DEFICIENT PATIENTS BEFORE AND AFTER USE OF LYOPHILIZED AMINO ACIDS INTRAVENOUSLY

PATIENT	TOTAL QUANTITY OF AMINO ACIDS (GM.)	DAYS OF ADMINISTRATION	PLASMA VOLUME (CC.)	TOTAL SERUM PROTEIN (GM. %)	TOTAL SERUM ALBUMIN (GM. %)	TOTAL CIRCULATING ALBUMIN (GM.)	TOTAL CIRCULATING ALBUMIN (GM.)	HEMATOCRIT (%)	WEIGHT CHANGE (KG.)
1	570	10	Before 2375 After 1700	5.2 6.5	3.6 4.3	123.5 111.4	85.2 73.0	38.0 38.5	-1.8
2	1520	16	Before 2114 After 2710	6.1 5.6	3.7 4.6	128.7 152.8	79.5 124.6	37.5 39.0	-2.0
3	722	16	Before 2237 After 2983	6.3 5.7	4.1 3.9	203.9 170.5	132.4 116.7	38.0 35.0	-2.0
4	1606	16	Before 1925 After —	6.1 4.3	3.2 2.7	170.5 118.2	116.7 61.5	35.0 45.0	-2.0
5	904	14	Before 3268 After 2000	4.2 4.6	2.7 3.6	140.0 139.9	89.8 81.0	34.0 17.5	+3.1
6	1606	16	Before 1604 After 2407	5.1 5.8	3.6 3.6	81.0 140.0	51.2 86.3	35.0 35.0	+2.0
7	1280	17	Before 2912 After 2889	5.5 5.1	3.4 3.0	163.0 150.0	98.8 86.7	30.0 29.0	-1.9
8	1551	17	Before 3215 After 3424	5.8 5.2	4.6 3.9	189.7 178.1	147.8 133.5	26.0 26.0	-0.5
9	442	7	Before 2283 After 2103	5.1 5.1	2.2 2.3	116.4 114.0	50.3 50.8	35.0 35.0	+3.2
10	898	13	Before 2551 After 1927	5.8 5.3	3.5 3.0	149.0 102.0	89.2 57.5	36.0 32.0	+0.8
11	1668	20	Before 3096 After 2433	5.6 6.4	2.9 3.5	173.7 156.4	89.6 85.2	40.0 41.0	-2.1
12	1240	17	Before 2604 After 2422	5.0 4.6	2.2 2.1	130.2 111.4	57.1 49.8	30.0 28.0	+0.8
13	975	15	Before 2933 After 2710	6.2 6.3	3.7 3.5	141.5 173.4	108.4 94.7	36.0 37.0	+1.0
14	798	16	Before 2049 After 1905	7.0 6.7	4.1 4.0	140.8 121.0	84.0 76.2	26.0 32.0	-3.2
15	765	14	Before 2222 After 1961	6.0 5.9	3.3 3.2	133.0 116.0	73.3 62.9	33.0 39.0	+0.9
16	1302	15	Before 2703 After 2807	6.3 5.8	4.5 3.7	170.0 166.0	121.6 107.7	45.0 39.0	+1.0
17	744	12	Before 2174 After 2800	5.9 6.4	3.5 3.6	127.0 154.0	76.1 104.4	40.0 32.0	+1.9
18	735	12	Before 1970 After 2400	6.0 5.6	3.5 3.8	113.0 137.0	60.5 91.2	34.0 35.0	-0.8
19	1014	12	Before 2381 After 2273	5.1 5.2	1.3 1.4	74.0 73.0	31.0 31.8	35.0 35.0	+1.2
20	104	5	Before 2526 After 2817	4.9 4.7	— —	124.0 131.0	58.1 67.0	35.0 33.0	+1.8

Table III also shows that the patients showed no significant weight change during the period of study. Twelve patients showed gains in weight up to 3 kilograms, but the remaining eight showed losses of equivalent quantities. Both the gains and losses were probably chiefly due to changes in body water content, for in all cases the caloric intake was little more than a maintenance one.

#### DISCUSSION

The data presented here offer unequivocal evidence that the lyophilized casein hydrolysate used in these studies on patients with chronic protein deficiency is a nutritionally and clinically adequate product. The material can be reconstituted easily with water, glucose solution, or physiologic saline to form a clear solution that is intravenously injectable without reaction. Positive nitrogen balance has been obtained in nineteen of the twenty patients studied. The quantities required for nitrogen equilibrium, though varying from an unusually low value of 42 mg per kilogram per day to the very high value of 349 mg, averaged 129 milligrams. This means that the average person is likely to obtain his daily nitrogen requirements with an injection of about 60 Gm, or one bottle of these amino acids.

But the adequacy of this protein preparation was determinable in this study by the method of minimal requirements for positive nitrogen balance only by virtue of the large number of balance experiments performed. A random selection of four or five patients might have given values for minimal requirements for equilibrium of the order of 50 mg per kilogram per day, or they might have averaged 200 milligrams. In the former case the parenterally injected amino acid mixture could have been regarded as superior to the best natural protein mixture given orally. If the latter values had been obtained the product would have been regarded as unsatisfactory. The range was found to be wide in spite of the choice of what was regarded as a homogenous group of patients with chronic protein deficiency and in spite of rigidly controlled regimens with adequate caloric intakes and with the same foods for all patients.

As in Allison's experiments with dogs, the nitrogen intake at equilibrium has been found to be dependent upon the nitrogen excretion on a protein-free diet. If the latter is small, equilibrium is established at a low intake. In general, this means that patients with long-standing protein deficiency require much less nitrogen for equilibrium than patients who have had recent acute losses, as has been found by Browne and co-workers.<sup>11</sup> Yet it was impossible to correlate endogenous nitrogen excretion in these chronically ill patients with the serum protein concentration or total circulating protein. Apparently the exact level of the circulating proteins is determined by many factors rather than merely by the degree of protein deficiency. Apparently, also, there is no easy clinical or laboratory method of anticipating the requirements for equilibrium other than nitrogen balance studies.

The finding of rectilinear curves in the region of negative and low positive balance in thirteen of the nineteen subjects offers proof of the applicability of the concepts of biologic value of proteins as expounded by Mitchell,<sup>12</sup> Allison

Terioine,<sup>13</sup> and others to the study of a parenterally injected protein product in protein deficient human subjects. The relative constancy of the  $K$  value of these curves—the nitrogen balance index of Allison—in the face of variations in nitrogen intake requirements for equilibrium makes the determination of that value the better gauge of the adequacy of the protein derivative under study. The average value of 0.68 for the nineteen cases or 0.66 for the more reliable thirteen cases indicates a good protein. Allison found values of about 0.80 for orally administered casein and of 0.39 for a soybean protein. We have data<sup>14</sup> which indicate that the protein hydrolysate used by us has a much higher biologic value when administered orally. Such a finding is to be expected in view of the fact that the higher plasma amino acid concentrations achieved with intravenous administration promote a greater destruction of amino acids and an increased urinary loss. Thus with oral administration of this product the  $K$  value might approach the value of 0.80.

Yet the use of the nitrogen balance index to characterize a protein as satisfactory as it is theoretically is not without its weaknesses in the study of protein deficient patients. In the first place it requires a long experiment of twenty or more days. In such protracted studies with an unattractive diet and with daily intravenous infusions it is difficult to maintain a constant level of cooperation of the patient and of his emotional reaction toward the dietary management. Besides, his clinical condition and therefore his protein requirements are likely to change during the course of the study. Second the accuracy of the curve depends primarily upon the establishment of the endogenous nitrogen excretion. This is not easy to determine accurately. When protein is removed from a calorically adequate diet the nitrogen excretion rapidly diminishes and is usually much lower on the third or fourth day than on the first or second. It has been necessary at times to discard the values of the first and second days. Smith<sup>15</sup> found that the endogenous nitrogen excretion had not become stabilized even after twenty-four days of a nitrogen poor diet. Third the slope of the curve depends upon the accuracy of a number of points. The variable errors of the changing condition of the experiment, temperature variations, excretion losses, and analytic defects may combine to produce a succession of points for which no accurate rectilinear curve can be drawn. The number of experiments must be large to avoid the consequences of these inaccuracies.

Though these experiments were not designed for the study of the therapeutic value of parenterally administered amino acids, they offer some corroboration of our previous findings<sup>1, 2</sup> that improvement of patients with long standing protein deficiency requires an intense and protracted program which is difficult to achieve with parenteral administration alone. Unless the biologic value of hydrolysates is markedly increased by the addition of individual essential amino acids the quantity of such amino acids that must be administered and the length of the program are too great to be clinically practicable.

The leveling of the curves of nitrogen balance on the high intakes indicates the approach of a ceiling of nitrogen retention. If the retention of each additional increment is no more than 10 or 20 per cent it becomes doubtful whether

the additional injections are worth the effort and the cost. Undoubtedly, however, there are many patients with severe protein deficiency who must be prepared for operation in as rapid a time as possible at whatever cost. In these instances the intravenous administration of large quantities of amino acids supplemented with whole blood and with as high an oral intake of food as possible might produce the desired results.

#### SUMMARY

Nitrogen balance studies were carried out in twenty patients with chronic protein deficiency. The experiments were performed with progressive increases in the nitrogen intake, the only significant source of nitrogen being a new parenterally injected lyophilized casein hydrolysate.

The lyophilized product could be injected without reaction in concentrations up to 10 per cent at reasonable speeds for periods up to twenty days.

Positive nitrogen balance was achieved in nineteen of the twenty subjects.

The nitrogen intake required for nitrogen equilibrium varied from 42 to 340 mg per kilogram per day. The average was 120 mg, with a standard deviation of 51 per cent. The values were directly proportional to the magnitude of the endogenous nitrogenous excretion.

In thirteen of the nineteen cases the curves of nitrogen balance were rectilinear in the region of negative and low positive nitrogen balance, in the remaining six, straight-line curves could be drawn but were not so well defined. In the region of high positive balance the curves tended to level off, indicating a ceiling of utilization. These findings are in corroboration of those of Allison for protein-starved dogs.

Allison's nitrogen balance index ( $K$ ), which is the slope of the rectilinear curve of nitrogen balance, tended to be constant in all nineteen cases, averaging 0.68, with a standard deviation of 24 per cent. In the thirteen more precise cases,  $K$  averaged 0.66, with a standard deviation of 20 per cent. The nitrogen balance index was therefore found to be a more reliable indication of the quality of the protein under study than the nitrogen intake requirement for equilibrium. The latter can be used only when a large number of balance studies are carried out with a homogeneous group of subjects as in this study. By either criterion the results indicate that the lyophilized casein hydrolysate is the equivalent of a good protein.

The determination of the nitrogen balance index has the following disadvantages: (1) the requirement of a long, tedious, difficult balance study, (2) the inherent error of the estimation of endogenous nitrogen excretion, and (3) the difficulty in drawing an accurate straight-line curve.

Serum protein concentrations or total circulating protein levels were not appreciably increased during the balance studies. A much more intense and protracted regimen is required to alleviate severe chronic protein deficiency.

We wish to express our gratitude to Mrs. Irene Antonow Maxwell who as dietician prepared all menus, and to Miss Lorraine Schmelzle and Mrs. Jacqueline Schaefer for the chemical analyses.

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TABLE I DAILY AND TOTAL DOSES OF CYTOCHROME C

SUBJECT	DAILY DOSE (MG)	TOTAL DOSE (MG)
Normal	50 500	978
1 (Als)	50	800
2 (Pma)	512	512
3 (Pma)	100	1200
4 (Mg)	50 100	1400
5 (Als)	50 500	2750
6 (Pma)	50 500	3050
7 (Pma)	100 200	2825
8 (Md)	50 100	4320

Als Amyotrophic lateral sclerosis pma progressive muscular atrophy mg myasthenia gravis md muscular dystrophy

almost continuous recordings of these functions for three hours following injection. The cytochrome c solution also could be administered intramuscularly without causing undue pain or any reaction.

Total doses of 512 to 4,320 mg cytochrome c injected intravenously in daily doses which varied from 50 to 500 mg were administered to eight patients suffering from various neuromuscular diseases and to one normal control. These

TABLE II DETECTABLE CYTOCHROME C

DOSE (MG IV)	SERUM			URINE			
	(MIN AFTER INJECTION)			(HOURS AFTER INJECTION)			
	0	0	60	1	6	6	24
50	0	0	0	0	0	0	0
500	0	0	0	++	+	0	0
500	++	+	0	+++	++	+	0

injections appeared to be entirely innocuous but produced no detectable changes in symptoms or signs. None of our patients have yet shown any improvement attributable to cytochrome c.

From three of the patients and from the normal subject samples of blood were taken before, shortly after and twenty four hours after cytochrome c injections. The substance was not detected spectroscopically in the serum of these subjects except where blood samples were collected within thirty minutes after injecting single doses of 500 milligrams. Cytochrome c added to serum or water in vitro and reduced with hydrosulfite could not be detected with the hand spectroscope at concentrations of less than about 65 mg per cent. This concentration would correspond approximately to a dose of 325 mg in a total blood volume of 5 liters. One could not therefore expect to detect the cytochrome after smaller injections without more sensitive instruments. A pinkish color of the serum was observed in several samples but this was found to be due to hemolysis of red blood cells. The bands of oxyhemoglobin were readily seen spectroscopically but no bands of reduced cytochrome c could be seen on reduction with hydrosulfite. A solution of cytochrome c sufficiently strong to give an obvious pinkish color to serum or urine was found to contain about 20 mg per cent a concentration which is readily detectable with the hand spectroscope.

Samples of urine examined within thirty to ninety minutes after injection of doses less than 200 mg cytochrome c did not reveal the presence of this sub



stance When the doses injected were from 200 to 500 mg, the samples of urine, passed during the first few hours after injection, ranged from faint rose to deep copper in color, and the characteristic band of reduced cytochrome *c*, ranging from a faint thin line to a broad black band, was visible spectroscopically without the addition of reducing agent \*

#### CONCLUSIONS

The pinkish color of serum observed by Proger and Dekaneas<sup>1, 2</sup> after injections of cytochrome *c* may have been due to the presence of hemoglobin as a result of slight hemolysis Cytochrome *c* could be detected in serum by means of a hand spectroscope only immediately after large injections Considerably greater concentrations of the pigment are required to produce an obvious pink color

Injected cytochrome is rapidly removed from the plasma and considerable amounts are excreted in the urine

Intravenous injections of large amounts of cytochrome *c* produce no obvious effects While harmless, such injections were of no benefit in the few cases of neuromuscular disease studied

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\*The cytochrome injected was in the oxidized form but the ascorbic acid normally present in the urine would reduce the pigment Oxidized cytochrome *c* when added directly to urine is immediately reduced If the samples of urine were allowed to remain in open container oxidation occurred The urine turned pale-yellow and the cytochrome *c* band could no longer be detected However the rose or copper color and the band invariably reappeared even in samples which had stood at room temperature for more than three weeks on dissolving a few crystals of sodium hydrosulfite in the urine

## CARONAMIDE PLASMA CONCENTRATIONS, URINARY RECOVERIES, AND DOSAGE

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SINCE Caronamide† inhibits the enzyme transport system of the renal tubular epithelium by which penicillin is excreted,<sup>1</sup> the desirability of controlling dosage by accurate measurement of caronamide in the body fluids is obvious. When the initial studies with caronamide were carried out,<sup>2</sup> methods for determining caronamide plasma concentrations were not available. Dosage schedules were established by comparing penicillin dose response curves modified by caronamide with curves obtained on the same patients when penicillin was administered alone. On this basis it was found that from 9 to 12 Gm of caronamide per day (15 to 20 Gm every four hours) were sufficient to obtain a positive effect in some patients while others required 24 and even 32 Gm per day (30 to 40 Gm every three hours). The observation that different individuals required different dosages to bring about the same effect and the findings of others<sup>4</sup> that age apparently influenced the size of the dose required to give the maximal result, clearly indicated the need for methods for determining caronamide in body fluids in order that dosage could be properly individualized.

It is the purpose of this paper to report the plasma concentrations following various doses of caronamide by the oral and parenteral routes of administration, some recoveries of caronamide in the urine and the correlation of simultaneously determined caronamide and penicillin plasma concentrations. The results of this work permit a clearer definition of the effective dose of caronamide.

### *Methods for Caronamide Determinations—*

**Method 1** This method,‡ requiring that a forty eight hour alkaline dialysate of plasma be analyzed with the Beckman ultraviolet spectrophotometer proved to be more time consuming and less accurate than Method 3 and was abandoned as soon as the latter procedure became available. It was used, however during the initial phases of this investigation, and results obtained thereby have been cited previously.<sup>5</sup> Values obtained in this manner show substantial agreement with those obtained by Method 3 since both procedures measure the sum of caronamide and its modified forms.

**Method 2** The Brodie method<sup>6</sup> measures only free caronamide and not its conjugates. Acidification of urine or plasma precipitates caronamide which then is dissolved in chloroform. The chloroform solution is extracted with 0.1N sodium hydroxide solution and the resulting alkaline solution of caronamide is read in the Beckman ultraviolet spectrophotometer at 280  $\mu$  millimicrons.

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†4-Carboxyphenylmethanesulfonamide supplied as Statilin caronamide by Sharp & Dohme Inc., Glenolden, Pa.

‡This method was worked out by Mr. J. L. Cimniera, Department of Pharmaceutical Chemistry, Medical Research Division, Sharp & Dohme Inc., Glenolden, Pa.

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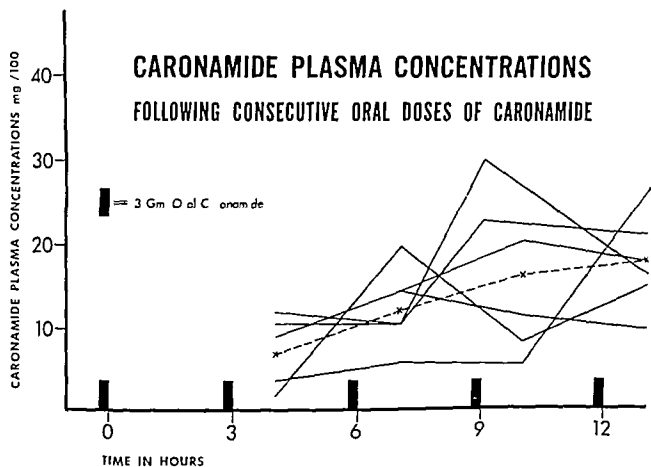
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\*The cytochrome injected was in the oxidized form but the ascorbic acid normally present in the urine would reduce the pigment Oxidized cytochrome *c* when added directly to urine is immediately reduced If the samples of urine were allowed to remain in open containers oxidation occurred The urine turned pale-yellow and the cytochrome *c* band could no longer be detected However the rose or copper color and the band invariably reappeared even in samples which had stood at room temperature for more than three weeks on dissolving a few crystals of sodium hydrosulfite in the urine

response curves and it is observed that, even in this small group, there were marked differences in handling of the drug. The average values obtained from estimation of caronamide plasma concentrations of the six patients were 15.5 mg per 100 cc after one half hour and 8.0 mg per 100 cc of plasma at the end of three hours.

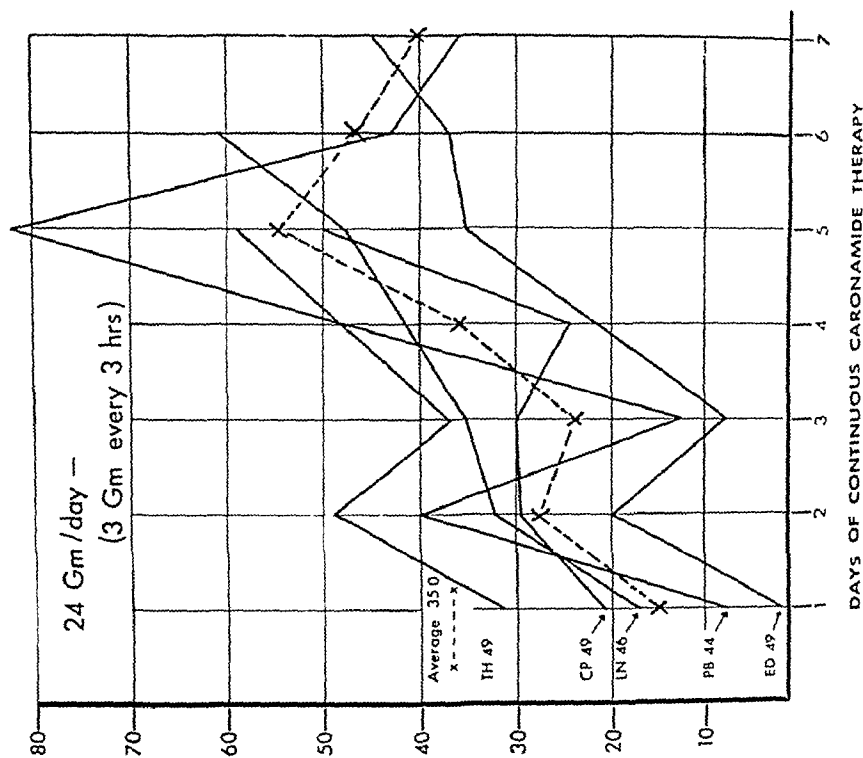
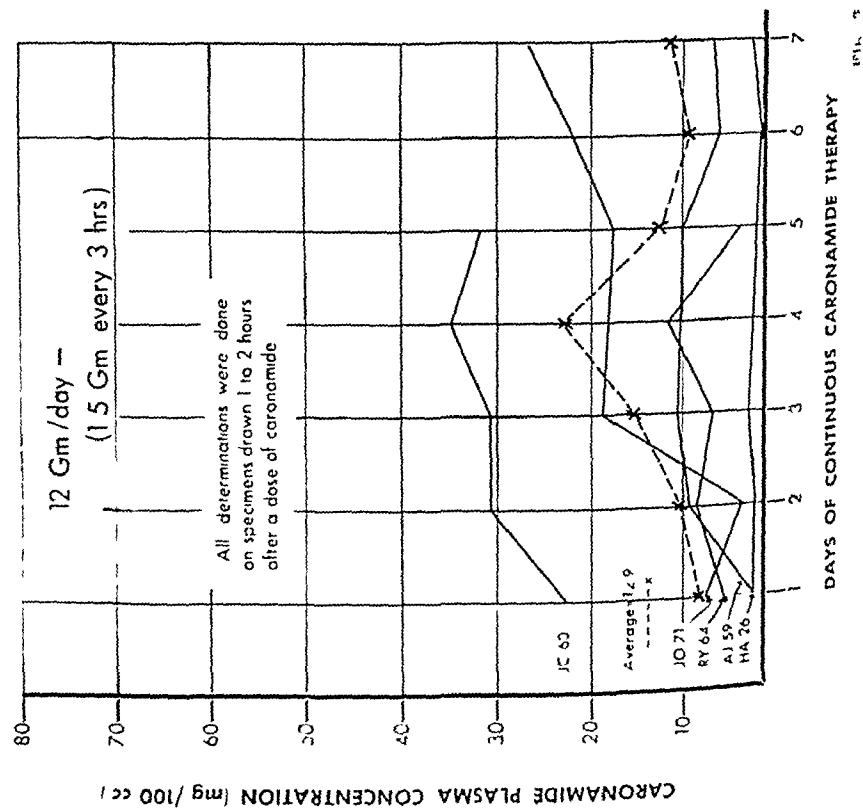
*Repeated Oral Doses of Caronamide*—Six patients, chosen at random in order that they might better represent the clinical conditions under which caronamide is likely to be administered, were given 3 Gm of the drug at three hour intervals for five doses. One hour after all but the first dose a blood specimen was drawn for caronamide determination. From the averaged results (Fig 2) it is clear that over the period of study there was a gradual rise in caronamide plasma concentration from 6.5 to 18.5 mg per 100 cc of plasma. All of these patients tolerated the drug well and no untoward symptoms were observed.



Fig

Since there appeared to be some tendency for the plasma concentration to rise gradually during a twelve hour period following initial administration of caronamide a similar study was conducted but observations were extended for a longer time. Five patients were given 15 Gm of caronamide every three hours and five patients received 30 Gm every three hours for one week. Several patients left the hospital before completion of the study and did not receive the drug for the full week. A plasma caronamide determination was made daily the blood specimen being drawn at approximately the same time each day from one to two hours following a dose of caronamide. Determinations on the plasma of Patient L N were estimated by Method 1 all the other results presented

# CARONAMIDE PLASMA CONCENTRATIONS\* FOLLOWING CONTINUOUS ORAL ADMINISTRATIONS



in Fig 3 were obtained by Method 3. Since Methods 1 and 3 both measure caronamide and its modified forms, however, the results are considered together.

Despite marked individual differences in the handling of the drug, the difference between the plasma concentrations of patients on the two dosage schedules is clearly defined. The averaged data on the patients who received 3 Gm of caronamide every three hours show a rising concentration of the compound. As noted in Fig 3 the caronamide determinations were done on blood specimens drawn either one or two hours after a dose of caronamide. From dose response curves following a single dose of caronamide it has been observed that the one hour determinations are uniformly higher than those done two hours after medication. In consequence some of the tendency of the average figures in Fig 3 to rise over the seven day period may be more apparent than real. That this is probable is confirmed by observations that during more prolonged administration of caronamide increasing plasma concentrations did not occur.

*Since Intravenous Dose of Caronamide*—Although one of the chief advantages of caronamide is the fact that it is administered orally, some experiments with intravenous caronamide have been done.

Three patients were given a 75 per cent solution of sodium caronamide intravenously, 3 Gm being injected over a period varying from three to seven minutes. All of these patients experienced a mild sensation of warmth after 15 to 20 cc of the solution had been injected but otherwise there were no reactions. At the same time 200 000 units of penicillin were injected intravenously in order that it might be determined whether or not the plasma concentrations of caronamide were sufficient to produce an elevation of the penicillin plasma concentrations as compared with those obtained in a control period without caronamide. In all instances a marked elevation of the penicillin concentration was noted (particulars of these experiments will be reported in another publication), indicating that the concentrations of caronamide (Table I) were effective in inhibiting penicillin excretion. Observations were limited to an hour and forty minutes and during this period quantitative urine collections were made making possible the measurement of caronamide in the urine (Table I).

TABLE I CARONAMIDE PLASMA CONCENTRATIONS AND URINARY RECOVERIES FOLLOWING A SINGLE INTRAVENOUS DOSE OF CARONAMIDE

PATIENT	AGE	IV DOSE OF CARONAMIDE (GM)	PLASMA CONCENTRATIONS OF CARONAMIDE (MC/100 CC)				RECOVERY IN URINE	
			40 MIN	60 MIN	80 MIN	100 MIN	(GM)	(%)
D S	41	3	10.0	8.75	7.20	0.21	1.15	3%
K S	34	3	12.30	9.62	8.24	6.25	1.45	48
T H	38	3	15.15	10.90	7.56	6.70	.66	21.9
Average							1.08	35.9

All determinations done by Method (Brodie<sup>6</sup>)

*Continuous Intravenous Infusion of Caronamide*—Three patients in apparent good health and with normal renal function received 3 Gm of sodium caronamide intravenously as a priming dose (injection time three to five

minutes) and thereafter were infused with a solution containing sodium caronamide (Table II). The actual amounts of caronamide administered were 5.04, 4.95, and 4.3 Gm, respectively, to Patients I S, M F, and B B. The caronamide plasma concentrations that were noted following these doses are given in Table II. These determinations were made by employing Method 2.

TABLE II CARONAMIDE PLASMA CONCENTRATIONS FOLLOWING CONTINUOUS INTRAVENOUS ADMINISTRATION OF CARONAMIDE

PATIENT	AGE	CARONAMIDE		CARONAMIDE PLASMA CONCENTRATIONS* (MG/100 CC)			
		IRRIGATING DOSAGE (CC)	INFUSION (MG/KG/HR)	45 MIN	60 MIN	75 MIN	90 MIN
I S	44	3	2.1	25.9	26.6	23.0	22.6
M F	17	3	3.2	21.7	23.0	18.0	25.4
B B	39	3	23.2	20.60	18.81	19.15	18.50

\*All determinations done by Method 2 (Brodie<sup>6</sup>)

*Recoveries of Caronamide After Oral and Intravenous Administration*—Five patients (five of the patients listed in Fig. 3) were given caronamide every three hours for five days and on the sixth day, while the drug was still being administered, twenty-four hour urine specimens were collected. The total amount of caronamide in this collection was determined both gravimetrically and by Method 3. For the gravimetric determination caronamide is precipitated by acidifying the urine to a pH below 5.0 and thereafter the precipitate is washed, dried and weighed. By this method only caronamide is measured, since its modified forms remain in solution. Method 3 measured the modified forms of caronamide in solution and hence the values obtained are higher than those representing only caronamide (Table III).

TABLE III RECOVERIES OF CARONAMIDE FROM TWENTY-FOUR HOUR URINE COLLECTIONS

PATIENT	AGE	CARONAMIDE (GM/24 HR)	24 HR URINE VOL (CC)	CARONAMIDE IN URINE		RECOVERIES (%)	
				(GM/100 CC)*	TOTAL*	GRAVI- METRIC	COLORI- METRIC
R O	71	12	1490	0.1245	1.805	15.04	42.0
H A	26	12	1275	0.7153	9.020	75.1	75.3
P B	44	24	1300	0.3754	5.632	23.4	43.0
L N	46	24	2300	0.5071	11.853	49.3	51.0
E D	49	24	1390	0.2485	3.404	11.1	25.0
Average						35.39	47.26

\*All values represent the average of duplicate aliquots

*Correlation of Caronamide Plasma Concentrations With Penicillin Plasma Concentrations*—Dosage schedules for caronamide originally were set up without the benefit of caronamide plasma concentrations by determining the dose of caronamide that influenced favorably plasma concentrations of penicillin. When methods became available for determining caronamide in body fluids the dosage schedules previously established were re-evaluated.

Penicillin dose-response curves modified by caronamide were compared with penicillin dose-response curves obtained on the same patients when penicillin alone was administered. For each curve the dose of penicillin was the same and, when the curve modified by caronamide showed at least a twofold

# CORRELATION OF CARONAMIDE AND PENICILLIN PLASMA CONCENTRATIONS

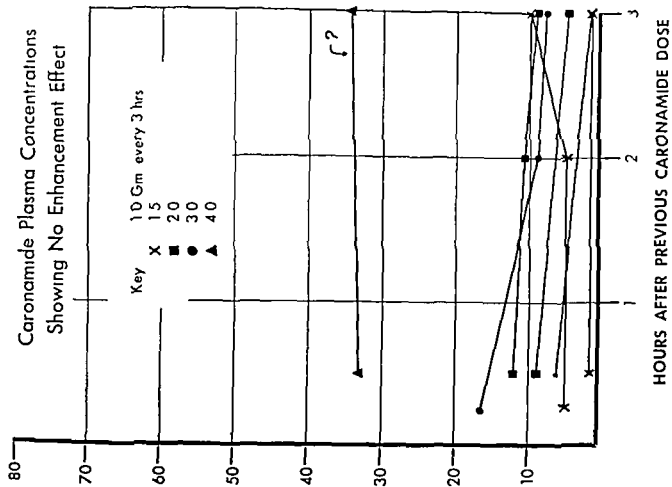
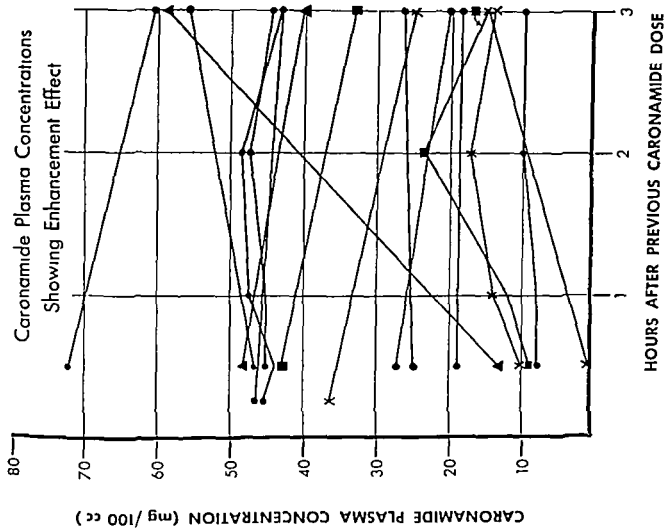


Fig. 4 (Boger W P Caronamide A New Enhancing Agent for  $\gamma$  e in Conjunction With Penicillin Therapy Tr & Stud Coll Physl  
*Class Philadelphia 15 104 1,4*)



enhancement over the control curve, the result was regarded as satisfactory. On this basis, caronamide plasma concentrations were correlated with penicillin plasma concentrations (Fig 4).

From Fig 4 it is apparent that a caronamide plasma concentration of 1 mg per 100 cc approximates the critical value that determines the inhibition of the excretion of penicillin. The striking exception presented has been questioned because there was very real doubt that the patient received the prescribed amount of penicillin. The criteria used to determine the positive and negative effects of caronamide demand that the patient receive the same dose of penicillin in the control period and the caronamide treatment period. If this requirement is not fulfilled, a failure of caronamide may be implied where none exists.

#### DISCUSSION

It has not yet been determined whether or not the metabolic products of caronamide are physiologically active. For this reason there would appear to be some advantage in using a method for determining caronamide that measures both the drug itself and its conjugates. Methods 1 and 3 fulfill this requirement, but because of its simplicity Method 3 is preferred.

Method 2 measures caronamide alone and, when aliquots of the same specimen have been analyzed by Methods 2 and 3, the values of Method 2 have been roughly half of those obtained by Method 3. Despite this relationship it would be hazardous to depend on it for not all patients conjugate caronamide to the same degree. The urinary recoveries of caronamide in only a small group of patients (Table III) showed that patients such as H A and L N either do not conjugate the drug at all or do so to only a slight degree. Other patients excreted almost half the caronamide as a conjugate or metabolic product of caronamide. It is apparent, therefore, that values obtained by Method 2 will not bear a constant relationship to values obtained by Method 3. Application of the two methods in the study of a larger series of patients should give valuable information concerning the metabolism of caronamide.

It has been noted previously<sup>8</sup> that a reducing substance occasionally appears in the urine following caronamide administration. Nine of the ten patients presented in Fig 3 showed this reducing substance in the urine. The exact nature of this substance has not been determined, but it reduces Benedict's solution, is still present in specimens subjected to reasting, and gives a positive Bial's test. The last mentioned test generally is performed to determine the presence of a pentose. It is interesting to note that a reducing substance has been observed in the urine of persons treated with para-aminobenzoic acid<sup>9</sup> and it is not unlikely that this reducing substance is the same as that observed following the administration of caronamide.

The demonstration that caronamide is effective in elevating penicillin plasma concentrations from two to seven times led to the conjectures whether the intervals between intramuscular injections of penicillin could be lengthened and whether caronamide might prolong the period of activity of a single intramuscular dose of penicillin in aqueous solution to equal the duration of action

of an injection of penicillin in oil and beeswax. The duration of action of a single dose of caronamide and the plasma concentration of caronamide required to inhibit penicillin excretion are intimately related to these questions.

At the beginning of investigations with caronamide clinical evaluation was handicapped by the inability to determine the compound in the plasma. Nevertheless it was found that a single 4 Gm dose of caronamide exerted an effect on penicillin plasma concentration for from five to seven hours. Later when methods for caronamide assay were developed it was found that a slightly larger single dose 4.5 Gm provided plasma concentrations between 8 and 15 mg per 100 cc (Method 1) for a period of three hours.

These plasma concentrations in man are in good agreement with the theoretic figure of 6 mg per 100 cc that is regarded as giving a good suppressive effect upon penicillin excretion in dogs.<sup>11</sup> Further the finding that the oral administration of a single 4 Gm dose of caronamide, that is approximately 50 mg per kilogram of body weight for an average adult weighing 70 kilograms (154 pounds) elevated plasma penicillin concentrations for from five to seven hours, confirms the finding in dogs that a single oral dose of 50 mg per kilogram that is 1 Gm for a dog weighing 20 kilograms decreased penicillin clearance for at least four hours.<sup>10</sup>

Since 4 Gm of caronamide as a single oral dose elevated penicillin plasma concentrations for from five to seven hours and a slightly larger dose (4.5 Gm) of sodium caronamide given orally resulted in a plasma concentration of 8 mg per 100 cc (Method 2) for only three hours it is suggested that even lower concentrations can partially inhibit penicillin excretion.

Reasoning from the foregoing, a dose of 4 Gm every four hours would maintain a plasma concentration of caronamide close to the one required definitely to inhibit penicillin excretion. Using this schedule of dosage it has already been shown<sup>4</sup> that 100,000 units of penicillin in aqueous solution will sustain assayable penicillin levels in the plasma for as long as eight hours. It is doubtful whether caronamide doses can be spaced farther apart than every four hours since it is excreted rather rapidly and it seems desirable to maintain an adequate caronamide plasma concentration in order to assure at least a two fold increase in the level of penicillin.

It has not yet been determined whether by continued administration of caronamide it will be possible to prolong the effects of a single injection of aqueous penicillin to equal that of penicillin in oil and beeswax. However if assayable levels can be obtained for as long as eight hours after 100,000 units of aqueous penicillin when caronamide is given the likelihood is great that with larger doses of penicillin assayable levels can be extended to at least twelve hours. To prolong the effect of a single injection of penicillin and to circumvent the necessity of relying upon the patient to take additional medication single oral doses of 6 Gm of caronamide have been given in conjunction with 500,000 units of oral penicillin. This treatment has been applied to a group of patients with acute gonorrhea and the rate of cure has been 87 per cent.<sup>11</sup> Caronamide plasma concentrations following the administration of 6

Gm are not available at this time but, in consideration of the results obtained following a 4 Gm dose, it might be anticipated that 6 Gm of caronamide would produce a concentration that would influence penicillin plasma levels for at least six to ten hours. Apart from the inconvenience of swallowing the tablets, 6 Gm doses have been given without unfavorable reactions and investigation of large single doses of caronamide is indicated.

In Fig 3 the impression is given that a fixed dose of 3 Gm every three hours may cause an accumulation of the drug in the plasma. That this rising concentration may be more apparent than real has already been mentioned. It is worth while to point out, however, that we have had the opportunity of observing other patients not reported here who have taken 3 Gm every three hours for two and three weeks without showing either increasing plasma concentrations or symptoms of systemic toxicity. An occasional patient has complained of nausea and a few have vomited. It has been difficult to differentiate between simple nausea induced by the difficulty incident to swallowing a number of large tablets and that caused by drug toxicity. It has been interesting to observe that a number of these patients have been able to continue medication at the same dosage level when a suspension of caronamide replaced the tablets. However, several instances of vomiting probably due to drug toxicity have been encountered, and in two of these patients it has been shown that caronamide plasma concentrations were in excess of 70 mg per 100 cubic centimeters. This finding only emphasizes the desirability of controlling dosage by caronamide plasma determinations. It should be pointed out that caronamide concentrations of 45 mg per 100 cc and above generally are well tolerated (Figs 3 and 4).

In a previous publication,\* on the basis of only two patients, it was suggested that 20 to 30 mg per 100 cc (Method 1) was the caronamide plasma concentration required to suppress the elimination of penicillin. On the basis of a larger experience and more reliable caronamide determinations, it appears that concentrations as low as 8 mg per 100 cc (Method 2) partially inhibit penicillin excretion and concentrations between 10 and 20 mg per 100 cc insure at least a twofold enhancement of penicillin plasma concentration (Fig 4). Concentrations between 20 and 40 mg per 100 cc are well tolerated and they approximate the plasma concentration of caronamide that provides optimal inhibition of penicillin excretion.

The dose that has most frequently fulfilled the requirement of maintaining for the entire time between doses of caronamide a plasma concentration that is adequate to inhibit penicillin excretion has been 3 Gm every three hours or 4 Gm every four hours. In a few instances this dose has failed to maintain the desired plasma concentrations and in others (Fig 3, Patients J C and J O, and Fig 4) 15 Gm every three hours have maintained the requisite level. Actually, the optimal dose of caronamide cannot be defined arbitrarily as a certain number of grams per day, the dosage necessary to maintain the plasma concentration at or above 15 mg per 100 cc varies from individual to individual and varies somewhat even in the same individual from day to day.

Diet, degree of hydration, fluid intake, fever, and disease influence to a marked degree the daily requirements of any particular patient. The avail

ability of a simple and reliable method for caronamide determination has established this clearly. Striking differences in individual requirements under conditions other than those just mentioned are probably a reflection of the patient's renal function. It appears that the more nearly normal renal function is the larger the dose of caronamide required to maintain a plasma concentration that will inhibit penicillin excretion. This generalization is compatible with the available clinical data. Children with normal renal function have been found to require daily doses equal to adult doses 24 Gm per day,<sup>1</sup> in order to obtain elevations of penicillin plasma concentration. Persons over 60 who, presumably on the basis of aging alone have some renal impairment require less caronamide to inhibit penicillin excretion than do younger individuals.<sup>4</sup> Caronamide probably is excreted only by glomerular filtration and, consequently impairment of glomerular function will retain the drug in the circulation, thus making smaller doses more effective. Furthermore since the site of action of caronamide is the renal tubule any existing impairment of tubular function means that less drug will inhibit those tubules that are functioning normally. Thus if a patient to whom caronamide is administered has subclinical renal impairment, a relatively small dose of the drug will be required to maintain an optimal plasma concentration.

#### CONCLUSIONS

A caronamide plasma concentration of approximately 15 mg per 100 cc is required to inhibit the renal excretion of penicillin sufficiently to provide at least a twofold elevation of penicillin plasma concentration. A single oral dose of 4 Gm of caronamide will not maintain this concentration in the plasma but by reason of partial inhibition of the renal tubules will, nevertheless, influence penicillin excretion for from five to seven hours. Although 15 Gm every three hours in some patients provide this critical level (15 mg per 100 cc) in the plasma, 3 Gm every three hours or 4 Gm every four hours are required in the majority of patients with normal renal function. Caronamide plasma concentrations of 20 to 40 mg per 100 cc are well tolerated and probably represent the concentrations that maximally inhibit the tubular excretion of penicillin. There are marked individual differences in the metabolism of this compound but the average twenty-four hour recovery of free caronamide is 35.38 per cent and of caronamide and its metabolic products 47.26 per cent of the material injected. The availability of a simple and reliable method for caronamide determination permits individualization of caronamide dosage.

We wish to acknowledge our indebtedness to Dr. Harrison F. Flippin and Dr. William G. Leaman, Jr. for having granted permission to study patients on their services at the Philadelphia General Hospital, Philadelphia, Pa.

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## ISOLATION AND IDENTIFICATION OF INFLUENZA VIRUSES DURING THE EPIDEMIC OF DECEMBER 1945

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THE ease with which influenza viruses can be isolated and identified from individuals ill with clinical influenza has varied considerably in different epidemics in different laboratories during the same epidemic and even in different patients studied in the same laboratory during the same outbreak. During the epidemic of influenza A which occurred in Boston in December 1943, and in sporadic cases that occurred during the ensuing weeks, successful virus isolations were made quite readily from the throat washings of a small group of patients with clinical influenza and from the lungs of patients with fatal cases.<sup>1</sup> These isolations were accomplished by using unfiltered and untreated throat washings or lung suspensions either for intranasal mouse inoculation and passage or for inoculation and subsequent passage through the allantoic sac of developing chick embryos or by combinations of these methods. Bacterial contaminations were usually eliminated in the course of the mouse passages without resort to special methods, or by filtration after the virus had multiplied and become established either in the mouse lungs or in the allantoic fluid of the chick embryo. Similar methods were used successfully during the same epidemic by workers in Minnesota.<sup>2, 3</sup>

The same methods, except for the addition of adequate amounts of penicillin to suppress bacterial contaminations, were entirely without success when used on materials collected from patients during and after the epidemic of influenza B that occurred in and around Boston in December 1945. After numerous attempts, influenza B viruses were eventually isolated from throat washings of a number of patients studied during this epidemic utilizing other routes of inoculation of embryonated eggs both with and without preliminary mouse passage.<sup>4</sup> In view of the difficulties encountered and the different methods employed it may be of interest to present here some of the details of the experiences with the isolation of influenza viruses during this outbreak. The results have been reported elsewhere of the serologic studies made in cases and family contacts from a localized outbreak in a school in one of Boston's suburbs,<sup>4</sup> in cases of uncomplicated respiratory infections,<sup>5</sup> and in patients with pneumonia<sup>6</sup> seen at the Boston City Hospital during this epidemic.

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\*Only three strains had been successfully established at the time that the report was made on the serologic studies in cases and family contacts from the school outbreak in Needham and only one of those strains was from a Needham case. Several other strains were subsequently isolated on one or more attempts both from children in Needham and from patients at the Boston City Hospital as detailed in this paper.

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dissected out, pooled ground with sterile alundum and made up to a 10 to 20 per cent suspension with broth containing 10 per cent normal horse serum. This suspension was used for further egg passages when the allantoic fluid failed to agglutinate the chicken cells.

*Allantoic Inoculations*—For this purpose 10 to 11 day old embryonated eggs in groups of four to six were inoculated directly into the allantoic sac in the manner described by Hirst<sup>8</sup>. A hole was drilled on the side of the egg over the embryo but in an area where there were no large blood vessels. A second puncture was made in the shell over the air sac. An inoculum of 0.1 ml. was then injected with a 26 gauge needle one half inch long through the side opening directly into the allantoic sac. Both holes were then sealed with the collodion iodine mixture. After two days of incubation at 35 or 37° C. depending on whether influenza B or A virus respectively was anticipated the allantoic fluid was harvested without previous chilling. The large blood vessels in the chorioallantois were ruptured and allowed to bleed into the allantoic cavity. The allantoic fluid containing the embryonic red cells was collected, pooled and concentrated according to the method of Francis and Salk<sup>10</sup>.

*Mouse Inoculations*—Mice in groups of six, each weighing 10 to 12 grams were inoculated intranasally under light ether anesthesia with 0.05 ml. of untreated throat washing. The mice were sacrificed on the third day, the lungs dissected out, and the presence or absence of lesions was noted. The lungs were then pooled, ground with sterile alundum and made up to approximately a 20 per cent suspension in 10 per cent horse serum broth. After centrifugation to remove coarse particles the supernatant fluid was removed and used for further passages.

*Controls*—Sterile horse serum broth and negative or unknown materials including throat washings and lungs from patients without clinical influenza were carried through all of the various egg and mouse procedures and yielded negative results in every instance. Care was taken not to work with established viruses on days when isolation procedures were being carried out.

*Serologic Tests*—The methods used for titrating the viruses and for the agglutination inhibition and complement fixation tests were similar to those used in previous studies reported from this epidemic<sup>4</sup>. Neutralization tests were carried out in chick embryos by the method of Hirst<sup>11</sup>. The PR8 and Lee strains\* have been maintained by allantoic passages in eggs. The BON strain<sup>12†</sup> was not introduced into the laboratory until most of the strains from this epidemic had already been isolated.

Identification and differentiation of the viruses were carried out in part by the use of antisera prepared in rabbits. For this purpose albino rabbits weighing 2.5 to 3.5 kilograms were used, two for each strain of virus. Each rabbit was bled about 10 ml. from the marginal ear vein to obtain serum for controls in subsequent tests and was then injected intravenously with 0.1 ml. of concentrated active virus. The rabbits were again bled from the ear vein after ten to fourteen days and the serum obtained at this time together with

\*Originally obtained from Dr. Thomas Francis, Jr., School of Public Health, University of Michigan.

†Obtained from Dr. John F. Enders, Department of Bacteriology, Harvard Medical School.



the controls was heated at 60° C for twenty minutes and tested for inhibition of chicken cell agglutination by the homologous virus. If there was a satisfactory rise in titer, the rabbits were bled by cardiac puncture and the serum collected and stored in sealed tubes at -20° C.

## RESULTS

*Virus Isolations*—Only fourteen throat washings obtained from patients with clinically typical cases of influenza were studied. Each of these washings, however, was used in from two to twenty-seven separate attempts to obtain virus by inoculation and passage utilizing various methods or combinations of methods. The source of the washings, the antibody titers of influenza A (PR8) and influenza B (Lee) in the serum of the patients from whom they were obtained, and the results of the various attempts to demonstrate the presence of virus in these washings are summarized in Table I.

Successful isolations were made on one or more attempts from eight of these fourteen throat washings. All of these eight washings were obtained between December 13 and 21 from patients who developed significant rises in titer of antibodies for influenza B and not for influenza A.\* The six which failed to yield any virus include all three of the washings that were obtained after the middle of January from patients who developed a rise in antibodies for the PR8 strain of influenza A and not for the Lee strain nor for any of the recently isolated strains of influenza B. Interestingly enough, two of the other three failures were with the latest washings obtained from patients with serologic evidence of influenza B. The serums of two of these patients, W D and J C, showed, in addition fourfold or greater rises in antibodies to three strains (MB VF, and WC) of influenza B that were isolated from this epidemic. The washings from J C, however, were not carried through the usual blind passages in eggs. Of all the six washings which failed to yield a virus, only the one from W D was obtained on the third day of illness, the others were collected on the first or second day.

With the washings that yielded viruses, the same methods were not always equally successful. There were not enough observations made with each method or combination of methods to permit definite comparisons. The available data nevertheless seem to warrant some deductions as to the relative efficacy of the different methods. The amniotic route of inoculation was definitely the most successful for the direct isolation of the viruses in eggs. In no instance could virus be established if washings were inoculated initially by the allantoic route. Inoculation directly into the embryo was not used as often as the other methods and was somewhat more cumbersome but it seemed to yield results similar to those obtained by amniotic sac inoculations.

Mouse inoculation and passage was not successful alone in establishing any of the viruses. Indeed when typical lesions were present in the lungs of mice inoculated with the original washings the lesions usually became progressively

\*Since this paper was submitted successful isolations of influenza B virus were made from stored throat washings of Case 10 (J C) on three separate occasions. The virus was first recognized in Am<sub>1</sub> in one instance in M<sub>1</sub>A<sub>1</sub> in a second and in M<sub>1</sub>Am in the third. It was maintained through Am<sub>1</sub>A<sub>1</sub> the second through M<sub>1</sub>A<sub>1</sub>B<sub>1</sub> and the third through M<sub>1</sub>Am A<sub>1</sub>.

TABLE I RÉSUMÉ OF ATTEMPTS TO ISOLATE VIRUSES FROM PATIENTS WITH INFLUENZA  
DECEMBER 1945 TO JANUARY, 1946

NO	PATIENT	SERUM TITERS		DAY	DATE	VIRUS ISOLATION			
		LEE	PPS			ROUTE	RESULTS	ROUTE	RESULTS
1	M B	2/107	3/3	1	12/13	Am	4/6	M Am	1/1
						F	1/4	M E	0/1
						E	1b/1	M Al	0/2
						Al	0/3	M Am	1/1
						Al	0/1	M E	0/1
						Am Al	0/2	M Al	0/1
						Am Al	1/1	M Al	1/1
								M <sub>2</sub> Al	0/1
2	D C	-	--	1	12/13	Am	11/-	Am Al	1/1
						Al	0/3	M Am	1/1
						Al	0/1		
3	I M	10/44	11/11	3	12/13	Am	1b/1	Am Al	1b/1
						Al	0/1		
4	R T	4/256	14/16	3	12/13	Am	11/-	Al	0/1
						E	0/1	Am Al	1b/1
5	W D	14/512	9/6	3	12/13	Am	0/4	Al	0/1
						Am	0/1	Am Al	0/1
						E	0/-		
6	M F	4/256	14/16	1	12/14	Am	1/2	Am Al	1d/-
						L	2b/2	F Al	11/1
						Al	0/3	M Al	1m/1
						Al	0/1	M Al	1/1
	W C	1/44	-/2	-	12/14	Am	1/3	Am Al	1b/1
						Al	0/1	E Al	1/1
						E	0/1	M Al	1b/1
8	M M	9/143	7/8	1	12/14	Am	1/4	E Al	0/4
						E	0/3	M Am	11/1
						E	0/1	M Am	1/1
						Am Al	0/-	M Al	0/2
						Am, Al	1/1	M Al	0/1
9	F O	6/28	8/	1	12/14	Am	1/2	Am Al	0/1
						Al	0/1	Am, Al	1/1
						E	0/1		
10	I C	4/13	10/9	1	12/23	Am	0/-	M E	0/1
						E	0/-	M Am	0/1
						M Am	0/1	M E	0/1
						M Al	0/1		
11	D S	6/28	8/7	-	1/4	Am	0/1	Al	0/1
12	F M	44/44	20/10	-	1/19	Am	0/2	Al	0/1
						Am	0/1	F Al	0/-
						E	0/1		
13	I M	168/104	--/112	2	1/28	Am	0/3	F	0/1
						Am	0/1	Al	0/-
						E	0/2	Al	0/1
14	C R	10/11	2/48	-	1/31	Am	0/3	Al	0/1
						Am	0/-	Al	0/1
						E	0/2	Am Al	0/3
						E	0/1	E Al	0/1

Serum titers average titers of complement fixation in patient's serum acute/convalescent Day day of illness Date day when throat washing was obtained Route Am amniotic  
Embryonic Al allantoic Al suspension of lung from mouse inoculated intranasally sub  
scripts represent number of passages by that route Result number positive (good agglutination  
of hens cells)/number attempted

One was positive in Al and negative in Am fluid. Positive Al fluid maintained through E Al. Lost on subsequent passages. Positive Al fluid maintained through Am Al. Positive Al fluid passed through M Am lost in two subsequent Al passages. Passage through Am Al fluid from Am negative all others positive. Maintained through Am Al. Positive through Am Al fluid positive after Am. Maintained through Am Al. Al fluid positive both times. Passage through E Al. Passage through M Al with wide variations in titer. Nasal secretions inoculated passage through M Al. fluid in two attempts. Passage through Am Al. Passage through M Al. Passage through Am Al fluid positive after Am. Negative after Al passage positive on further Am passage. Positive Al fluid maintained on Am passage. Positive Am and Al fluids Am through Am. Passage through Am Al. Positive then lost in passage.

Numbers 1 to 7 are from Needham, the others are from Boston City Hospital

less marked or were not seen grossly after further mouse passages. On the other hand, one or more passages through mice seemed to result in a sufficient increase in concentration of virus to permit a better yield and more rapid establishment of the virus after subsequent amniotic passage in eggs. Preliminary mouse passage also permitted the establishment of the virus directly in the allantoic sac of the chick embryo on several occasions without preliminary amniotic passage.

A few details of interest and some of the difficulties encountered in the course of these isolations may be mentioned briefly. There seemed to be considerable fluctuations in the amount, or possibly the virulence, of the viruses during the course of the amniotic passages and before they were stabilized by subsequent allantoic passages. It was difficult in the early passages, for example to judge from the hemagglutination titers the dilution of amniotic or allantoic fluid to use for subsequent passage either by the amniotic or allantoic routes. It was necessary, therefore, to use two, three, and sometimes four different dilutions of fluid for injections by both the amniotic and allantoic routes in order to assure a good virus yield and to avoid the loss of the virus in passage. Furthermore, the detection of virus in allantoic fluid, even in moderate or high titer did not always assure subsequent successful allantoic passage even when several dilutions of virus were used. Eventually, however, it was possible to passage some of the viruses consistently by the allantoic route, although not all of the strains were carried through to that stage.

Storage of the throat washings or of passage materials in the carbon dioxide icebox did not seem to affect the viruses appreciably, at least as judged by the facility with which isolations were subsequently made from these materials. Viruses were isolated from original washings and first egg passage materials by two individuals working independently, as long as fifteen to eighteen months after they were originally stored.

One point of particular interest was the progressive decline in titer of virus in the amniotic fluid with a concomitant increase in titer of the virus in the allantoic fluid during the course of incubation of the amniotic passages. Three striking examples of this phenomenon are shown in Table II.

TABLE II VIRUS TITERS IN AMNIOTIC AND ALLANTOIC FLUIDS AT VARIOUS INTERVALS AFTER AMNIOTIC INOCULATION

PATIENT	SOURCE*	DAYS OF INCUBATION	CCA† TITR	
			AMNIOTIC FLUID	ALLANTOIC FLUID
E O	Am <sub>4</sub> (10 <sup>-2</sup> )	2	128	2
		3	<2	32
		4	<2	64
F O	Am <sub>4</sub> (10 <sup>-3</sup> )	2	128	16
		3	16	512
		4	2	32
R T	Am <sub>6</sub> (10 <sup>-1</sup> )	2	128	32
		3 (Alive)	16	64
		4 (Dead)	4	128

\*Am<sub>4</sub> and Am<sub>6</sub> Fourth and sixth amniotic passages respectively nine-day-old embryos used for these inoculations. The dilution of the inoculum from the previous amniotic passage is shown in parentheses.

†CCA Chicken cell agglutination.

*Identification and Differentiation of Strains —*

*Human Convalescent Sera* In the course of the serologic studies of the cases from the school outbreak in Needham,<sup>4</sup> some evidence was obtained which suggested that there were antigenic differences among the strains of influenza virus isolated in this epidemic although the strains were readily identified as influenza B. Most of the patients from Needham who had clinically typical influenza showed a characteristic rise in antibody titer in the convalescent sera with the Lee strain of influenza B as well as with three recently isolated strains but not with the PR8 strain of influenza A virus.

There were three patients in that group however who failed to show a rise in antibodies by the agglutinin inhibition test with the Lee strain and with one of the recently isolated strains but did show a rise with the other two epidemic strains that were used. In two of these three patients similar results were obtained with the complement fixation test, but in the third patient the complement fixation test showed similar rises in titer with all of these four strains of influenza B virus. The serologic findings in these three children and in the three patients from whom the viruses were isolated are shown in Table III.

TABLE III ANTIBODY TITERS OF SERA FROM SELECTED PATIENTS TESTED WITH STOCK STRAINS AND RECENTLY ISOLATED STRAINS

PATIENT	PR8		IFF		BON		WC		MF		MB	
	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT	ACUTE	CONVALESCENT
<i>Agglutination Inhibition Tests</i>												
W C	21	4	14	56	--	--	3	24	--	--	--	--
M F	2	4	11	115	2	16	2	40	5	64	2	64
M B	28	40	9	172	6	32	4	96	3	16	6	128
J H	16	20	3	4	3	16	2	16	2	16	2	2
R V	66	72	3	3	--	--	2	6	2	16	3	3
J M	48	48	6	6	--	--	2	4	6	16	4	4
<i>Complement Fixation Tests</i>												
W C	--	2	3	44	--	--	--	--	--	--	--	--
M F	2	3	4	272	2	48	3	180	6	192	3	80
M B	3	3	2	107	--	--	2	64	--	56	2	113
J H	10	8	6	8	2	24	2	12	3	10	5	7
R V	8	7	2	2	--	--	2	14	2	6	2	3
J M	11	11	10	44	--	--	10	32	10	40	12	48

Patients M B, J H, R V, and J M were 1, 14, 11, and 1 years old respectively and were from Needham. W C and M F were adult workers at the Boston City Hospital. Titers with homologous virus are shown in bold type.

On the basis of the meager evidence obtained from the serologic response in the three Needham patients, the strain MB that was isolated from another case in Needham appeared to be antigenically closely related to the Lee strain. On the other hand strains MF and WC which were isolated from individuals in Boston who had not been exposed to patients from Needham, differed from these two strains and were closely related to the Australian BON strain<sup>1</sup> of

influenza B. It is interesting to note that these differences in antigenic response to the different strains of virus were obtained in school children ranging in age from 11 to 17 years. No such specific differences in antibody response were noted in the convalescent sera of the adult patients from the Boston City Hospital.

The fact that only the convalescent sera from some of the school children showed these specific differences may be due to their having had fewer previous exposures to influenza antigens, particularly to those of type B strains. This may have permitted their antibody response to express greater specificity when compared with the antibody reaction of older patients.

*Immune Rabbit Sera* The titers of agglutinin-inhibiting antibodies in the rabbit sera prepared against these various strains are shown in Table IV. The results of these tests corroborate the findings in the human convalescent sera. They show that the MB strain is indistinguishable from the Lee strain of influenza B while strains WC and MF are identical with each other and are easily differentiated by this method from the first two viruses. The WC and MF strains furthermore, appear to be closely related to the BON strain. All of these strains were easily differentiated from the PRS strain of influenza A virus.

The titers of antibodies for the homologous virus in the rabbit sera obtained at the time of the final bleeding are also expressed in Table IV as fold rises. This was done because the rabbits used for immunization with the MF strain had a high titer (1:128) of hemagglutinin-inhibiting activity before the immunization. The subsequent fourfold increase in antibody titer in these rabbits was less than in those which were immunized with the other strains and in which the control titers were lower. The anti-MF serum was less active than the other rabbit antisera and the difference was even more marked in the results obtained in the virus neutralization tests with these sera.

TABLE IV. CHICKEN CELL HEMAGGLUTINATION INHIBITION TITERS OF ANTISERA PREPARED IN RABBITS AGAINST SPECIFIED STRAINS OF INFLUENZA VIRUS

VIRUS	TITER OF HOMOLOGOUS RABBIT SERA AT TIME OF FINAL BLEEDING			TITERS OF IMMUNE RABBIT SERA TESTED SIMULTANEOUSLY AT A LATER DATE					
	CONTROL	IMMUNE	FOLD RISE	PRS	LEE	BON	WC	MF	MB
PRS	4	2048	512	512	8	8	8	16	8
Lee	1	1024	256	8	64	8	16	16	64
BON	32	256	8	8	32	64	32	32	32
WC	16	256	16	4	16	16	128	64	16
MF	128	512	4	4	16	32	64	64	16
MB	16	256	16	16	128	8	16	16	128

Titers with homologous virus are in bold type

The results of the virus neutralization tests performed on embryonated eggs are shown in Table V. These tests appear to be more sensitive and more specific and, therefore, give a more accurate measure of the antigenic differences. In these tests the control or normal rabbit sera showed no neutralizing activity with any of the viruses. The results otherwise confirm the close antigenic relationships of strains MB and Lee and their differences from strains WC, MF, and BON which are closely related to each other. There are also minor but

suggestive antigenic differences between WC on the one hand and MF and BON on the other. These latter differences however may be due in part to the fact that the neutralizing titer of the antiserum prepared with the WC strain was appreciably higher than that of the MF and BON antisera.

TABLE V RESULTS OF VIRUS NEUTRALIZATION TESTS IN CHICK EMBRYOS WITH IMMUNE RABBIT SERA AND SELECTED STRAINS OF INFLUENZA VIRUS

VIRUS	IMMUNE RABBIT SERUM TITERS AGAINST					
	PR8	IFF	BON	WC	MF	MB
PR8	>128*	<4	--	<4	<4	<4
Lee	<4	>128	--	<4	<4	32
BON	--	8	16	16	12	<4
WC	--	12	--	64	16	<4
MF	<4	10	--	16	15	<4
MB	<4	64	--	<4	<4	20

\*Titer expressed as the reciprocal of the dilution of serum which inhibits growth in 10 per cent of embryos inoculated with 300 ID<sub>50</sub> (50 per cent infecting doses)

*Hemagglutination of Horse Cells* The ability of all of these strains of virus to agglutinate horse red blood cells was tested in order to detect any possible difference in activity similar to that found for some strains by Burnet.<sup>13</sup> There were some suggestive differences but these were not marked nor were they observed consistently in repeated tests.

*Attempts to Demonstrate a Receptor Gradient* Experiments were also performed to determine whether any possible differences in these viruses could be determined by the receptor gradient analysis as described by Burnet and co-workers.<sup>14</sup> Each of the viruses was used to agglutinate chicken red blood cells. The viruses were then eluted from the red cells. The cells from which each of the viruses was eluted were then used for agglutination with all the viruses. No appreciable differences were noted in the effect on the red cells of any of the strains of influenza B virus that were tested in this manner.

#### SUMMARY AND CONCLUSIONS

Details of the isolation and identification of influenza viruses from throat washings obtained from patients with clinical influenza in and around Boston during the epidemic of December, 1945 have been presented.

Strains of influenza B virus were isolated only during the height of the epidemic from patients whose convalescent sera showed a rise in antibodies for influenza B and not for influenza A.

The amniotic route of inoculation of chick embryos was the most successful for the primary isolation of these viruses. Preliminary mouse passage was sometimes helpful in establishing the virus more rapidly in the embryonated eggs.

Evidence was presented which indicated that at least two antigenically distinct strains of influenza B were active in this epidemic.

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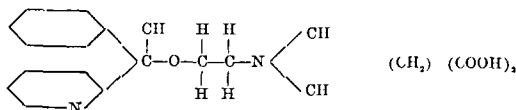
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## HISTAMINE ANTAGONISTS

### A NEW ANTIHISTAMINIC DRUG 2 [ $\alpha$ (2 DIMETHYLAMINOETHOXY) $\alpha$ METHYL BENZYL] PYRIDINE SUCCINATE (DECAPRYN SUCCINATE) EXPERIMENTAL AND CLINICAL RESULTS

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RAPID developments in the field of antihistaminic drugs have led to the synthesis of another new compound which, according to our experimental and clinical experiences, is an effective antihistaminic and antiallergic agent. This substance is 2 [ $\alpha$  (2 dimethylaminoethoxy)  $\alpha$  methylbenzyl] pyridine succinate, which the manufacturer calls Decapryn succinate.\*



Laboratory studies by Brown and Weiner<sup>1</sup> have demonstrated that decapryn succinate is an antihistaminic agent with comparatively high potency and low acute and chronic toxicity. Minimal doses necessary to antagonize 4 to 7 LD<sub>100</sub> of histamine were reported to be less than 0.5 mg per kilogram intravenously and subcutaneously and 10 mg per kilogram orally, lethal doses by these routes were sixty-four or more times the effective doses. Protective action in these tests was of long duration, as evidenced by the fact that seven lethal doses of histamine were antagonized ten hours after the oral administration of 80 mg per kilogram of the antihistaminic agent.

Decapryn succinate effectively antagonized the depressor response in cats and the pressor response in rabbits resulting from histamine. It was found to have practically no topical anesthetic action on rabbit cornea but alkalization of solutions liberated the base and resulted in strong local anesthetic action. The succinate also produced strong anesthetic action of long duration when injected with epinephrine. It prevented anaphylactic death in guinea pigs passively sensitized to horse serum, it antagonized the massive edema producing effects of intraperitoneally administered egg white in rats, and both oral and topical applications antagonized in some measure the contact dermatitis induced by chemical antigens.

These data indicated to us that decapryn had properties warranting further experimental and clinical study.

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## EXPERIMENTAL INVESTIGATIONS

Decapryn succinate produces very little surface anesthesia of mucous membranes. Unlike most of the other antihistaminic drugs, a few crystals on the tongue did not produce noticeable numbness. One or two per cent solutions in the rabbit's eye did not abolish the sensitivity of the cornea. However, the intradermal injection of a solution into the guinea pig indicated that it is a local anesthetic about equal in potency to novocaine.

*Intravenous Histamine*—The 1 MLD 100 of histamine (0.4 mg of histamine base per kilogram) given intravenously to guinea pigs resulted in a survival of ten out of ten animals when an intraperitoneal injection of 1 mg of decapryn succinate per kilogram was administered twenty minutes previously. When 0.1 mg per kilogram of the drug was injected, two of five animals died after 1 MLD 100 of histamine.

*Aerosols in the Prevention of Histamine Bronchospasm*—We have found that aerosols of the antihistaminic drugs were effective in the prevention of dyspnea from aerosolized histamine to which the guinea pigs were subsequently exposed. Ten animals were selected which responded to our histamine aerosol (0.5 mg per cubic centimeter at 5 pounds pressure) with the production of labored breathing in one-half to three minutes. These same animals were subjected on different days to the antihistaminic drug, usually in strengths of 2, 1,  $\frac{1}{2}$ , and  $\frac{1}{4}$  per cent for a five-minute period. After fifteen minutes the animals were exposed to the histamine aerosol and the time required to produce dyspnea was noted. If no dyspnea occurred after ten minutes the animals were removed. Periodically the animals were tested for changes in histamine tolerance and, if changes were noted, were replaced with other animals.

TABLE I. EFFECT OF DIFFERING CONCENTRATIONS OF AEROSOLS ON GUINEA PIGS TOLERATING HISTAMINE AEROSOLS (TEN ANIMALS WITH A NORMAL HISTAMINE TOLERANCE OF ONE HALF TO THREE MINUTES)

DRUG (%)	NUMBER OF ANIMALS TOLERATING DOUBLE HISTAMINE EXPOSURE	NUMBER OF ANIMALS TOLERATING 10 MIN HISTAMINE EXPOSURE
<i>Pyribenzamine</i>		
2	10	10
1	10	10
$\frac{1}{2}$	10	9
$\frac{1}{4}$	6	1
<i>Decapryn succinate</i>		
2	9	6
1	5	1
$\frac{1}{2}$	5	0
$\frac{1}{4}$	7	
<i>3015 RP</i>		
2	8	3
1	2	0
$\frac{1}{2}$	3	0
$\frac{1}{4}$	0	0
<i>Antistine</i>		
2	10	1
1	6	1
$\frac{1}{2}$	5	0
$\frac{1}{4}$	11	0

The essential results with decapryn succinate in comparison with Pyribenzamine and two other drugs are briefly reported in Table I. It may be seen that while decapryn succinate as an aerosol is not so effective as some antihistaminic drugs, it is more effective than others.

*Anaphylaxis*—Some degree of protection against anaphylaxis was evidenced by the fact that of nine animals passively sensitized with antiegg serum and challenged with 0.5 cc of 10 per cent egg white intracardially only one died of anaphylaxis when an intraperitoneal injection of 3 mg of decapryn succinate per kilogram was used thirty minutes previously. With a 1 mg per kilogram dose the protection was practically nil. The control group showed a mortality of 85 per cent.

*Inhibition of Local Histamine Skin Response in Man*—Early in our experience with the antihistaminic drugs we demonstrated that when applied locally to skin scratches these substances were highly effective in inhibiting the wheal and flare of histamine from subsequent application. Since it is reasonable to suppose that the histamine skin response in man comes much closer than animal assays to simulation of the mechanism of the allergic phenomena which we attempt to ameliorate it was hoped that quantitative studies of the inhibiting action of this histamine reaction might be a valuable means of obtaining a preliminary assay of the drug in question. At this point we shall not go into detail concerning the various techniques and the modifications which we employed nor

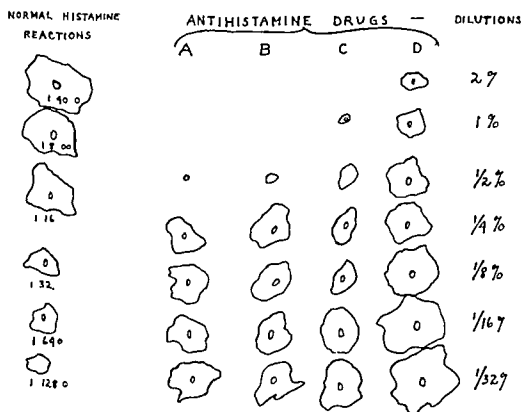


Fig 1—Quantitative inhibition of histamine reaction. Suitability and sensitivity of the particular subject are determined by preliminary titration of histamine (dilutions applied to the site of scratches on the back) (column at left). Dilution of antihistaminic drugs—A Pyribenzamine N (pyridyl) N benzyl N dimethyl ethylenediamine hydrochloride B Decapryn succinate C Chlorothal N (dimethyl N (pyridyl) N (2-chloro-2-phenyl) ethylamine hydrochloride D Hetramine N (dimethyl N benzyl N (2-pyrimidinyl) ethylenediamine hydrochloride—are applied to several rows of scratches on the same subject at the same dilution. The drugs are washed off ten minutes later and 1 drop of a selected concentration of histamine (in this case 1:1000) is applied to each of the scratches. The comparative inhibitive effect of the drug gives a good indication of its antiallergic efficacy. The procedure is repeated on a number of subjects. Other modifications of the technique based on the same concept are being used.

discuss our general data with respect to other drugs. We shall outline here one method which can be used to obtain data and also the results obtained with decapryn succinate.

The suitability and the sensitivity of the subject are determined by performing tests with serial dilutions of histamine on scratches on the back. The dilutions commonly employed range from 1/4,000 to 1/128,000 in terms of histamine base. The concentration which is twice that of the lowest giving the maximum flare is usually employed in the inhibition experiments. Several series of scratches are made on the back. On each series of scratches drops of an antihistaminic substance of serial dilutions are placed, the range varying from 2 per cent to 1/32 per cent\*. After ten minutes the antihistaminic solutions are washed off and the selected concentration of histamine is applied to the pretreated sites. Inhibition of the histamine reaction is shown in the sites which had the more concentrated antihistaminics, while the sites which had the weak solution may react. The point at which a histamine reaction fails to be inhibited gives an idea of the antihistaminic and probably the antiallergic potency of the drug. The experiment is, of course, repeated on a number of subjects.

Fig. 1 gives an idea of a representative result and indicates that decapryn succinate is effective in the inhibition of histamine reactions.

#### CLINICAL FINDINGS

*General Procedure*—After some of the experimental work had been done, the drug was administered cautiously to several subjects. When no particular toxic effects were noted decapryn succinate began to be used for the symptomatic relief of allergy. The patient was given the drug in the form of capsules or tablets and instructed to use small doses at first and only when needed. In a few instances the medication was prescribed to be taken regularly two to four times daily. In most instances the patient was seen twice weekly, when his condition was evaluated by questioning and observation and judged in terms of pollen counts, weather changes, and similar factors. Whenever feasible the subject was asked to compare the action of this drug against one or more others at various times. The results were regarded as satisfactory if relief was consistently obtained, although not necessarily every time. If relief was very slight or questionable or not obtained most of the time the result was recorded as "no improvement."

*Hay Fever and Nonseasonal Allergic Rhinitis*—The seasonal group comprised eighty-one patients, observed during the 1947 season, having seasonal hay fever either due to the pollens of grasses or ragweed or to fungus spores. Subjects with hay fever due to ragweed predominated. While some of the patients to whom this drug was given were new or untreated, the majority had received previously varying amounts of desensitizing injections. Satisfactory relief was obtained in sixty-two of the persons with hay fever (76 per cent).

\*More recently we have found the following factors more suitable: histamine range of 1/8,000 to 1/512,000 for control titration; concentration of histamine for inhibition either the first effective flare or one which is one-half size of maximal; and antihistamine concentration ranging from 1/800 to 1/204,800.

The relief was, of course, only temporary. The duration of action is difficult to determine in hay fever, our impression however is that there was a tendency to longer action than found with other antihistaminic drugs.

In the nonseasonal vasomotor rhinitis, nineteen of thirty four patients obtained satisfactory improvement. In both the seasonal and nonseasonal types of rhinitis the hyperesthetic symptom of sneezing was more often relieved than the nasal blocking. The hypertrophy of the turbinates, so common a manifestation of the latter part of the hay fever season was resistant to this drug as well as to other antihistaminic drugs.

*Other Allergic Manifestations*—No appreciable relief could be observed in any of twenty seven patients with asthma. Most of the patients with asthma in this group were associated with hay fever. This fact cannot be overstressed. In hay fever asthma, the hay fever may be effectively relieved whereas the asthma is not. Since desensitization treatment is highly effective in the prevention of such asthma, it is obvious that antihistaminic therapy should not be depended upon in such cases. Two patients of seven had relief of allergic cough.

The itching and some of the swelling of urticaria and angioneurotic edema were relieved in six patients of nine treated. Six patients with chronic atopic dermatitis received decapryl succinate. Four had appreciable relief of itching. Dermographism was effectively relieved in five of six patients. Two patients with headache of possible allergic origin failed to obtain benefit.

*Doses*—The doses administered varied from 12.5 to 50 milligrams. We found no one who failed to tolerate as little as 12.5 mg. and doses larger than 50 mg. usually either were not needed or were not tolerated well. It is true that some patients who failed to obtain relief from 50 mg. doses might have obtained benefit from larger amounts. It is also true that some of those who obtained results from 25 mg. doses without toxic effects may have been relieved by smaller doses. This is a phase of therapy which will have to be decided in the future. However it is well to emphasize here that decapryl succinate has in some patients a highly soporific action and large doses should not be prescribed to an individual without previous trial of smaller doses.

Of the sixty two patients with hay fever who obtained relief thirty did so with 25 mg. doses and 3 with 12.5 milligrams.

*Side Actions*—Of one hundred eighteen patients side actions to the drug were observed in thirty nine (34 per cent). Of this number sedation or sleepiness was seen in thirty six. In some patients this sedative effect was very pronounced. In one instance sleepiness and a feeling of numbness lasted from twenty four to thirty six hours. Usually however the sedative effect did not prevent the use of the drug. Nervousness was noted in four patients and vertigo also in four. Two patients complained of headache one of epigastric pain and another of an itching rash. No serious or remote toxic effects have been noted after six months use of the drug.

TABLE VI ANTISPASMODIC ACTIVITY OF 2 PS, ATROPINE, AND PAPAVERINE ON ISOLATED RABBIT JEJUNUM

COMPOUND	MINIMAL EFFECTIVE CONCENTRATION		
	NORMAL INTESTINE	VS ACETYLCHOLINE (1 1,000,000) SPASM	VS BaCl (1 10,000) SPASM
2 PS	1 50,000	1 50,000	1 100,000
Atropine	1 10,000,000	1 80,000,000	1 200,000
Papaverine	1 150,000	1 100,000	1 150,000

Antiacetylcholine action was further studied in cats anesthetized with Amytal Sodium. Intestinal activity was recorded by a tambour connected to a balloon inserted into the jejunum, with a water manometer in the system to regulate intestinal pressure. Blood pressure was recorded in the usual manner from the carotid artery. The intestinal responses were standardized by repeated intravenous injections of 0.01 mg of acetylcholine per kilogram, and no preparation was used unless identical consecutive responses were obtained. 2 PS was then injected intravenously and was followed by the standard acetylcholine injections at two- to four-minute intervals. 2-PS in a dose of 4 mg per kilogram antagonized acetylcholine action on the intestine by approximately 50 per cent. Twice this dose produced a slightly greater inhibition. The effect of 2-PS on the blood pressure response to acetylcholine was not evaluated since in these experiments the acetylcholine doses employed were far above minimal doses required for maximal responses in the blood pressure.

*Blood Pressure and Respiration*—Intravenous injections of 2 PS in cats anesthetized with Amytal Sodium, given at a rate of 2 mg per minute, produced a slight temporary pressor effect with doses of 2 mg per kilogram and either a slight pressor or depressor effect with doses of 4 and 8 mg per kilogram. A rapid rate of injection of 20 mg per minute produced a transient fall in blood pressure with doses of 8 mg per kilogram and a slight pressor or depressor response with 2 and 4 mg per kilogram.

Respiration generally was increased temporarily in both rate and depth with doses of 4 and 8 mg per kilogram regardless of the rate of injection. Smaller doses had no appreciable effect.

*Irritation*—2-PS produced no signs of irritation or tissue damage following either one minute instillation in rabbits' eyes of solutions up to 4 per cent or the injection of 1 per cent solution subcutaneously in guinea pigs and intramuscularly in rats. Subcutaneous and intramuscular injections of 3½ and 4 per cent solutions in rats produced irritation and some necrosis.

#### SUMMARY

Experiments in laboratory animals demonstrate that 2-[ $\alpha$ -(2-dimethylaminoethoxy)- $\alpha$ -methylbenzyl]-pyridine succinate (2-PS) has a comparatively low toxicity and has potent antagonistic action to the effects of histamine on various tissues.

It suppresses bronchial constriction resulting from intravenous injection of histamine in guinea pigs. Following intravenous administration, it antagonizes in average of up to 200 lethal doses of histamine and prevents death.

in some animals from 320 lethal doses. A high degree of antagonistic action also follows subcutaneous and oral administrations in guinea pigs, and the protective action is of long duration.

2 PS effectively antagonizes arteriolar constriction and pressor effects of histamine in rabbits and it also antagonizes the depressor action of histamine in rats. Effective doses of histamine for these two vascular effects are increased roughly fifteen to thirty fold.

Cutaneous effects of histamine, as measured by the whealing reaction in rabbits, are antagonized. The increased capillary permeability demonstrated by localization of intravenously injected dye is prevented for eight or more hours following a single dose of 2 PS orally.

2 PS has considerable local anesthetic activity and it is weakly antagonistic to acetylcholine.

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# THE EFFECTS OF ADRENALIN IN NORMAL AND HYPERTENSIVE PATIENTS IN RELATION TO THE MECHANISM OF SUSTAINED PRESSURE ELEVATIONS

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THE enigma of hypertension is not alone that the pressure rises but also that it fails to be restored to its former level. Many internal and external agents are capable of elevating the human blood pressure. Ordinarily homeostatic mechanisms induce a reversion to normal. The existence of sustained hypertension implies that the restorative mechanisms are either ineffective or in restraint.

One of the most important of these mechanisms is constituted by the modulator nerves, the interruption of which can produce a prolonged elevation in pressure. Investigations of the effect of carotid sinus stimulation by Weiss and Baker,<sup>1</sup> Moore and Allen,<sup>2</sup> and Thomas<sup>3</sup> have demonstrated that this mechanism is capable of greater depressor activity in the hypertensive person than in the normal person, indicating that sustained hypertension is not due to a reduction in the effectiveness of moderator action on peripheral structures. This capacity for vascular relaxation has been confirmed by a number of independent methods. Allen and co-workers<sup>4</sup> and Gregory and Levin<sup>5</sup> have reported that the pressure of hypertensive patients is reduced to values approaching normal by spinal anesthesia. Chasis and associates<sup>6</sup> induced prolonged remissions by utilizing the vasodilator action of parenteral pyrogens. More recently, the tetrathal ammonium ion like carotid sinus stimulation, has been shown by Beris and co-workers<sup>7</sup> to cause a pressure fall which is of greatest intensity in subjects with elevated pressure.

The concept of relatively ineffective homeostatic mechanisms is suggested by tests designed to show that hypertensive persons react excessively to pressor stimuli. Perhaps best known is the cold pressor test of Hines and Brown,<sup>8</sup> the use of which has been extended to single out prehypertensive individuals. However, exaggerated responses could not be demonstrated consistently in hypertensive patients by Pickering and Kissin<sup>9</sup> and were found absent in hypertension due to chronic renal disease by Miller and Bruger<sup>10</sup> and Alam and Smirk.<sup>11</sup> The use of the test to predict the subsequent occurrence of hypertension in pregnancy was attempted without success by Chesley and Chesley and Wellen.<sup>12</sup> An increased incidence of hyperreaction with age was demonstrated for both normal and hypertensive subjects by Russek and Zohman,<sup>13</sup> while marked variability of response on repetition was reported by Goldring and Chasis in studies conducted on the same subjects over a period of years.<sup>14</sup> The sum

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of these investigations indicates that a general relation between the level of blood pressure and the intensity of the cold pressor response has not been demonstrated.

The average pressor response to breath holding was reported by Ayman and Goldshine<sup>16</sup> to be two to four times greater in hypertensive persons than in normal individuals. However 20 per cent of their normotensive subjects were hyperreactors. Feldt and Wenstrand<sup>1</sup> compared the reactions to breath holding and cold in the same individuals. Correlation was absent in more than 25 per cent of the group.

The interpretation of the cold pressor breath holding and similar tests is complicated by the reflex nature of their action. As a consequence variations in the pattern of response cannot be ascribed primarily to differences in the capacity of the cardiovascular apparatus to compensate for a fixed stimulus but may be due rather to alterations in the intensity and duration of the discharge induced by the various types of reflex pressor stimuli.

The present study was designed to determine whether the pattern of response of the hypertensive person differed significantly from that of the normal individual when evoked by a pressor stimulus which bypassed the initial reflex arc. Adrenalin was chosen as the stimulus not only for its direct action on heart and vessels but also because of its rapid inactivation. It was decided to give the drug by continuous infusion at various rates since this type of administration allows better dosage control avoids differences due to rate of absorption and local sensory stimulation and facilitates equilibrium between the pressor actions of the drug and the depressor mechanisms of the subject.

Previous investigations of the action of adrenalin as modified by the initial pressure have been productive of varied results. The subcutaneous injection of 1 mg. was reported by Clough<sup>18</sup> to produce a greater incidence of excessive reaction in hypertensive subjects. However these reactions did not seem to be related to the type, extent or duration of the pressure elevation. On the contrary intravenous administration of single doses by Hougard<sup>19</sup> produced responses of the same magnitude in normal dogs as in those whose pressures had been significantly lowered by adrenalectomy. Continuous infusion of adrenalin at the rate of 2 mg. per hour was reported by Koehler and co-workers<sup>20</sup> to produce a rise in systolic pressure but a fall in diastolic pressure in both normal and hypertensive persons. Both the systolic rise and the postinfusion fall in pressure were said to be greater in subjects with elevated pressure.

#### EXPERIMENTAL METHOD

A total of eighty studies was carried out on thirty nine male and twelve female subjects whose blood pressures ranged from 90 to 246 mm. Hg. systolic and from 55 to 126 mm. diastolic. The age distribution is detailed in Table I.

Initial blood pressure and pulse values were established during the administration of saline through a three way stopcock connected to a calibrated drip apparatus. The stopcock was turned to permit infusion of the adrenalin solution from a flask suspended 200 cm. above the individual.

Pulse and blood pressure values were determined every three to five minutes during the course of the infusion every one minute for the first fifteen minutes after the infusion and at lengthening intervals throughout the next twelve hours.



TABLE I AGE DISTRIBUTION OF ADRENALIN INFUSION SUBJECTS

AGE (YR)	NUMBER OF SUBJECTS
30-39	6
40-49	15
50-59	7
60-69	12
70-79	11

Samples of blood were drawn for determination of the hematocrit value before infusion, again when the initial dose had produced its maximum effect, and finally at the point of minimum blood pressure following cessation of the infusion.

The adrenalin solution was made up immediately before use by diluting a standard U S P stock solution with isotonic saline to a final concentration of 10 mg per cent. A fresh bottle of the stock solution was used for each six patients. Normotensive and hypertensive patients were treated alternately. From time to time different stock solutions were checked against one another on the same patient. No significant variations in activity were noted.

The earlier subjects in the series were given adrenalin at an initial rate of 0.31 to 0.50  $\mu$ g per kilogram per minute. Later the initial rate was lowered to a value lying between 0.11 and 0.30  $\mu$ g per kilogram per minute since unpleasant side effects were encountered occasionally with the higher dosage.

When the starting level had produced its maximum effect on blood pressure, the rate of administration was increased in steps of 0.20  $\mu$ g per kilogram per minute until the limit of tolerance of the individual patient was reached. The infusion was maintained at this level until the end of a one and one quarter hour period.

*Tolerance and Untoward Effects*.—Tolerance was inversely proportional to the initial blood pressure. In the majority of instances, maximum tolerance was determined by the occurrence of unpleasant subjective reactions, including anxiety, tremor, heart consciousness and headache. One subject in the series complained of mild substernal pain which subsided without sequelae when the infusion was stopped. Cardiac irregularity, due to premature contractions, occurred to some degree in nearly all patients. In one instance it was sufficiently marked to dictate the cessation of the infusion.

Three persons developed cerebral symptoms, characterized in one by intense headache and momentary syncope, in another by transient unconsciousness, and in the third by a hemiplegic syndrome without loss of consciousness, from which recovery was complete in twelve hours. In two of these instances the reaction occurred during administration of the initial dose of adrenalin. These alarming episodes, which resembled the encephalopathic manifestations seen in pheochromocytoma,<sup>21</sup> occurred during the later phase of these studies and influenced their termination.

*Analytic Methods*.—The subjects were divided into four groups on the basis of the initial diastolic blood pressure level. The pressure range from 51 to 70 mm Hg included thirteen subjects, 71 to 90 mm, seventeen subjects, 91 to 110 mm, sixteen subjects, and 111 to 130 mm, five subjects.

The mean group values of the blood pressure, pulse rate, change in pressure and pulse rate, and the systolic diastolic pressure ratio were computed for each range of dosage tolerated by two or more of the subjects in a group. The pressure and pulse values which corresponded to the highest systolic pressure observed at each dose level were used in making computations.

Variability of response was estimated in terms of the standard deviation from the mean, corrected for small samples, while the significance of differences between groups were determined by calculation of the *t* value, which indicates the probability that the observed differences did not arise by chance.

The presence of linear relationship between variables was evaluated by calculation of the correlation coefficient.

## RESULTS

The sequence of events in the course of an adrenalin infusion is illustrated in Fig 1. During the first few minutes a period existed in which the incoming adrenalin solution was diluted by the saline in the tubing which connected the stopcock to the infusion needle. In this period the systolic pressure, diastolic pressure, or both ordinarily manifested a fall followed by a sharp rise when undiluted solution reached the circulation. This rise usually elevated the systolic and occasionally the diastolic pressure above the preinfusion value.

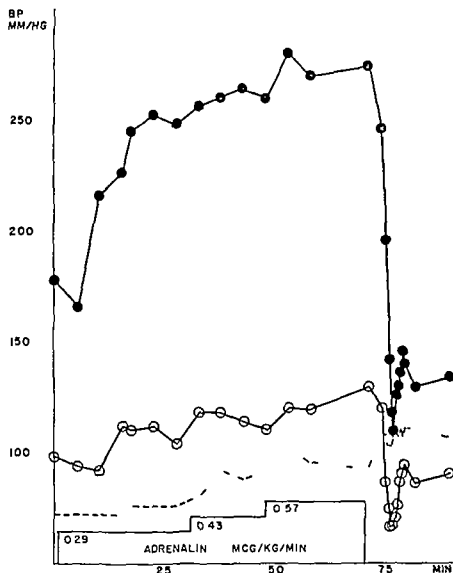


Fig 1—The effects of a continuous infusion of adrenalin on pulse rate and blood pressure (Subject J C). Solid circles systolic blood pressure open circles diastolic blood pressure dotted line pulse rate

An increase in the rate of administration produced a further rise in systolic and diastolic pressures to a new maximum in one to three minutes. The net result of successive increases in dosage was a steplike curve of pressure change.

In most instances infusion of adrenalin resulted in cardiac acceleration (Table II). The increase in rate usually was most marked at the lowest dosage range where it appeared roughly proportional to the initial pressure. Less than 25 per cent of subjects manifested at any time a fall in rate below the preinfusion value.



Following cessation of infusion both systolic and diastolic pressures dropped abruptly. A minimum level was reached usually by the tenth and not later than the fifteenth minute after clamping the infusion tubing. These minimum levels were lower than the initial blood pressure levels in all but one instance and were accompanied by cardiac acceleration.

*Results of Repeated Infusion*—Observations on the diminished effectiveness of chronic adrenalin administration in allergic disorders, coupled with reports of desensitization phenomena in patients treated for anxiety states by repeated adrenalin administration,<sup>2</sup> prompted a study of the effects of daily infusion.

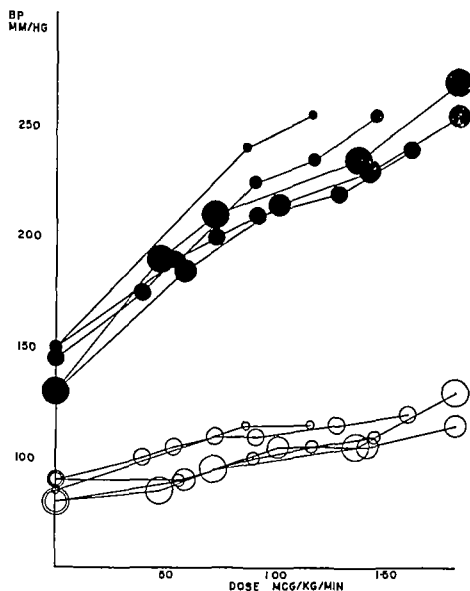


Fig. 1.—The blood pressure responses to daily infusion of adrenalin (Subject J. B.). Size of circle indicates temporal sequence. For clarity, the curves of every second day only are shown. Solid circles, systolic blood pressure; open circles, diastolic blood pressure.

Five subjects were used in this portion of the investigation. One was infused on twelve successive days, a second on ten, another on four, and two on three days. Despite some variation in the initial level of blood pressure from day to day, the slopes of the pressure response curves appeared almost identical within limits of the experimental method as illustrated in Figs. 2 and 3. For clarity, the curves of alternate days only are presented.

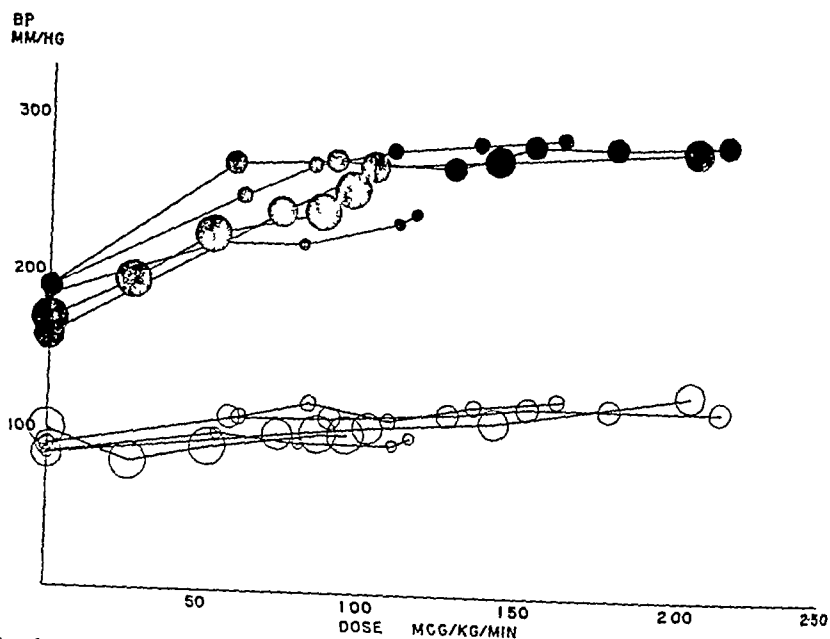


Fig 3 —The blood pressure responses to daily infusion of adrenalin (Subject J S) Size of circles indicates temporal sequence For clarity the curves of every second day only are shown Solid circles systolic blood pressure open circles diastolic blood pressure

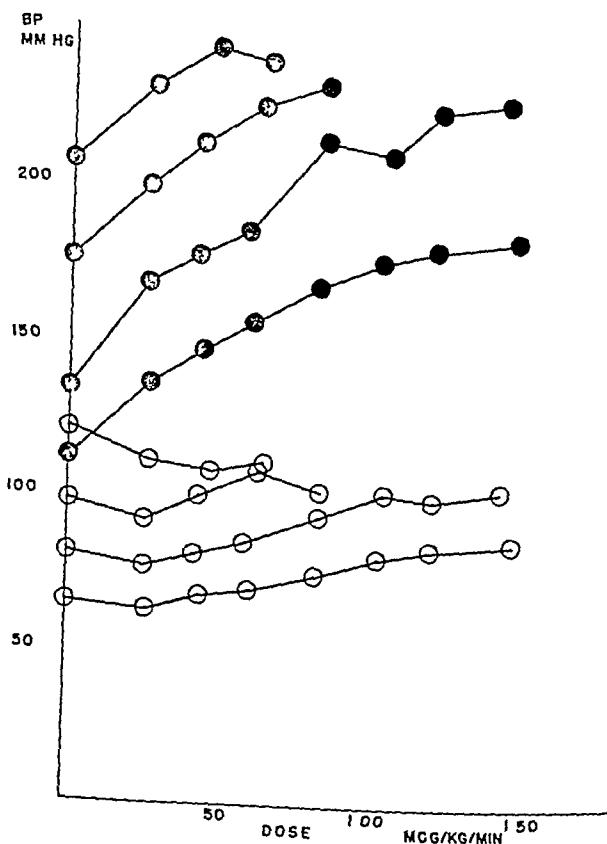


Fig 4 — Average blood pressure responses to adrenalin in subjects grouped on the basis of initial diastolic pressure. Solid circles systolic blood pressure open circles diastolic blood pressure

Although objective responses remained reasonably constant, increase in the subjective limit of tolerance was marked. The patient infused on twelve days accepted a dose on the last day which was ten times larger than that tolerated on the first day.

*Net Pressure Changes in Relation to Preinfusion Pressure*—The action of adrenalin on both systolic and diastolic pressures was characterized by a wide range of variation between individuals in the same group.

A mean rise in systolic pressure in all groups at all levels of dosage was produced as illustrated in Fig. 4. The pressure curves tended to parallel one another despite the differences in initial tension.

At the lowest level of dosage a mean fall in diastolic blood pressure was observed which was more marked the higher the initial pressure. Successive increments in dosage produced a rise in pressure at a rate roughly parallel in the four pressure groups.

The diastolic fall at low adrenalin dosages in the face of an increase in pulse rate suggested that systolic elevation was achieved through cardiac stimulation which overbalanced a compensatory drop in peripheral resistance. Such a drop in resistance might have resulted from moderator nerve activity alone or in conjunction with a direct dilator action of adrenalin. Whatever the nature of the compensatory mechanism, its value was limited to much the same extent in all groups as evidenced by the transition to diastolic elevation at dosages in excess of 0.30  $\mu\text{g}$  per kilogram per minute.

*Unit Pressure Changes in Relation to Preinfusion Pressure*—The rise in systolic pressure per microgram of adrenalin tended to correlate both with increase in pulse rate and with preinfusion systolic pressure over a restricted range of initial tensions, the upper limit of which lay between 180 and 200 mm Hg. However, when the unit systolic changes over the entire range were compared on a group basis (Table III, Fig. 5) the differences in mean response between groups did not prove significant. The  $t$  values for the differences ranged from 0.45 to 1.45.

TABLE III. CHANGES IN BLOOD PRESSURE IN RELATION TO INITIAL DIASTOLIC BLOOD PRESSURE AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

INITIAL P. RANGE	AVERAGE INITIAL SBP	A. CHANGES IN UNIT SYSTOLIC BLOOD PRESSURE (MM. HG/ $\mu\text{g}/\text{KG}/\text{MIN}$ ) AND STANDARD DEVIATION						
		RANGE OF ADRENALIN DOSAGE ( $\mu\text{g}/\text{KG}/\text{MIN}$ )						
		0.11-0.30	0.31-0.50	0.51-0.70	0.71-0.90	0.91-1.10	1.11-1.30	1.31-1.50
51-70	112	91 $\pm$ 57	81 $\pm$ 36	76 $\pm$ 31	70 $\pm$ 20	63 $\pm$ 14	59 $\pm$ 17	50 $\pm$ 9
71-90	134	137 $\pm$ 132	103 $\pm$ 65	90 $\pm$ 38	99 $\pm$ 73	74 $\pm$ 14	77 $\pm$ 13	67 $\pm$ 1
91-110	176	91 $\pm$ 86	93 $\pm$ 93	81 $\pm$ 48	69 $\pm$ 12			
111-130	207	59 $\pm$ 67	79 $\pm$ 46	54 $\pm$ 34				
range		106 $\pm$ 96	91 $\pm$ 62	80 $\pm$ 38	80 $\pm$ 17	69 $\pm$ 14	69 $\pm$ 16	57 $\pm$ 12
INITIAL P. RANGE	AVERAGE INITIAL DBP	B. CHANGES IN UNIT DIASTOLIC BLOOD PRESSURE (MM. HG/ $\mu\text{g}/\text{KG}/\text{MIN}$ ) AND STANDARD DEVIATION						
		RANGE OF ADRENALIN DOSAGE ( $\mu\text{g}/\text{KG}/\text{MIN}$ )						
		0.11-0.30	0.31-0.50	0.51-0.70	0.71-0.90	0.91-1.10	1.11-1.30	1.31-1.50
51-70	65	-11 $\pm$ 36	1 $\pm$ 23	9 $\pm$ 17	13 $\pm$ 12	16 $\pm$ 8	16 $\pm$ 7	15 $\pm$ 4
71-90	81	-14 $\pm$ 33	0 $\pm$ 28	8 $\pm$ 7	16 $\pm$ 10	21 $\pm$ 5	17 $\pm$ 9	16 $\pm$ 1
91-110	98	-23 $\pm$ 24	5 $\pm$ 34	10 $\pm$ 24	1 $\pm$ 22			
111-130	121	-40 $\pm$ 28	-28 $\pm$ 26	-15 $\pm$ 21				
range		-19 $\pm$ 33	-2 $\pm$ 29	9 $\pm$ 19	12 $\pm$ 13	17 $\pm$ 7	16 $\pm$ 8	15 $\pm$ 3

When the degree of activity at various dosages was computed, it became evident that adrenalin was decreasingly effective in its ability to elevate the systolic pressure as the rate of administration was increased. The over all unit response was halved with a sixfold rise in dose, while the maximum response which averaged 106 mm Hg per microgram, was observed at the lowest dosage range.

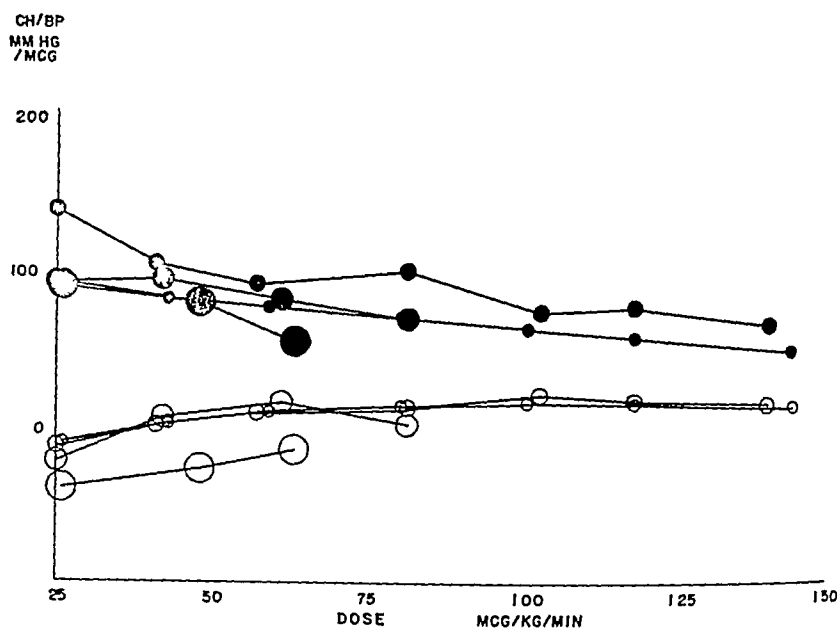


Fig. 5—Average unit blood pressure responses to adrenalin in subjects grouped on the basis of initial diastolic pressure. Size of circles indicates the relative magnitude of the initial pressure range. Solid circles, systolic blood pressure; open circles, diastolic blood pressure.

Comparison of changes in unit diastolic pressure (Table III, b, Fig. 5) demonstrated a fall at the lowest rate of administration which appeared inversely proportional to the initial pressure, but the coefficient of correlation ( $-0.229$ ) was not significant. Succeeding increases in rate resulted in a rise to a maximum value of 17 mm Hg per microgram at an average dose of  $1.0 \mu\text{g}$  per kilogram per minute, a value which showed little further change at higher dosage levels where a comparison between groups was possible.

After allowance was made for the wide range of individual variation there was evident a common pattern of reaction characterized by pressure changes similar in directions and magnitudes in each of the four pressure groups but different in the pressure base lines from which projected.

*Effects on the Systolic-Diastolic Pressure Relationship*—The similarity of the unit pressure changes in each group suggested that adrenalin was modifying some relationship between systolic and diastolic pressure which was largely independent of the absolute height of the pressure. To explore this possibility calculations were made of the correlation coefficient between systolic and diastolic pressures initially and at each dosage level. The value of the coefficient

(Table IV) averaged 0.80 which indicated the probability that the two pressures were related by a fairly constant factor. The magnitude of this factor was estimated by determining the systolic diastolic ratios at each rate of administration.

TABLE IV CORRELATION BETWEEN SYSTOLIC AND DIASTOLIC BLOOD PRESSURES AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

DOSE RANGE ( $\mu$ /KG/MIN)	CORRELATION COEFFICIENT
0	0.817
0.11-0.20	0.754
0.1-0.50	0.795
0.51-0.70	0.781
0.71-0.90	0.814
0.91-1.10	0.718
1.11-1.0	0.883
1.51-1.50	0.829
Average	0.800

The perfusion ratio averaged  $1.7 \pm 0.27$  and did not vary materially among the four pressure groups. The size of this ratio was somewhat surprising for its value at normal pressures is usually stated to approximate 1.5 while the increased peripheral resistance in hypertensive subjects is expected to exaggerate the diastolic pressure<sup>4</sup> and yield a ratio lower than normal. However, a calculation of this ratio from data reported by Steele<sup>5</sup> for a group of thirty nine patients whose blood pressures ranged from 94 mm Hg systolic, 60 mm diastolic to 262 mm systolic 164 mm diastolic gave an average value of  $1.72 \pm 0.10$ . Similar calculations were made from Goldring and

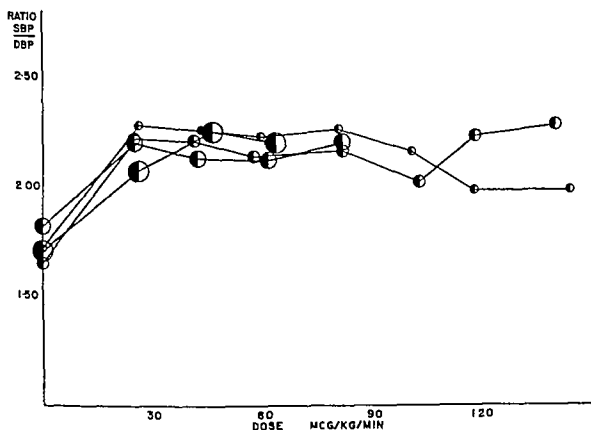


Fig. 6—Average change in systolic diastolic ratio produced by adrenalin in subjects grouped on the basis of initial diastolic pressure. Size of circle indicates the relative magnitude of the initial blood pressure range.



Chasis data<sup>1</sup> relative to fifty-six patients with mild to severe hypertension. The ratio of this latter group averaged  $1.59 \pm 0.19$  for both minimum and maximum recorded pressures.

At the lowest rate of adrenalin administration, the systolic diastolic ratio in the present series increased to an average value of  $2.21 \pm 0.38$ . This new value was also similar in all pressure groups and tended to remain constant with succeeding increments in dosage. These relations are illustrated in Fig 6 and their variability indicated in Table V. The *t* value (9.78) for the difference in ratio before and during infusion at the lowest dose range indicated the change to be highly significant.

TABLE V. SYSTOLIC-DIASTOLIC PRESSURE RATIO IN RELATION TO INITIAL DIASTOLIC BLOOD PRESSURE AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

INITIAL DBP RANGE	RANGE OF ADRENALIN DOSAGE ( $\mu\text{G}/\text{KG}/\text{MIN}$ )							
	0	0.11-0.20	0.21-0.30	0.31-0.40	0.41-0.50	0.51-0.60	0.61-0.70	0.71-0.80
51-70	$1.72 \pm 0.21$	$2.28 \pm 0.25$	$2.26 \pm 0.31$	$2.27 \pm 0.28$	$2.26 \pm 0.31$	$2.16 \pm 0.25$	$1.98 \pm 0.32$	$1.95 \pm 0.25$
71-90	$1.65 \pm 0.26$	$2.22 \pm 0.33$	$2.21 \pm 0.27$	$2.14 \pm 0.38$	$2.16 \pm 0.13$	$2.02 \pm 0.50$	$2.23 \pm 0.15$	$2.25 \pm 0.14$
91-110	$1.82 \pm 0.34$	$2.20 \pm 0.35$	$2.13 \pm 0.38$	$2.12 \pm 0.50$	$2.20 \pm 0.39$			
111-130	$1.71 \pm 0.07$	$2.67 \pm 0.07$	$2.25 \pm 0.13$	$2.27 \pm 0.12$				
Average	$1.73 \pm 0.27$	$2.21 \pm 0.38$	$2.21 \pm 0.37$	$2.17 \pm 0.37$	$2.22 \pm 0.26$	$2.10 \pm 0.30$	$2.12 \pm 0.24$	$2.10 \pm 0.24$

(Table IV) averaged 0.80, which indicated the probability that the two pressures were related by a fairly constant factor. The magnitude of this factor was estimated by determining the systolic diastolic ratios at each rate of administration.

TABLE IV CORRELATION BETWEEN SYSTOLIC AND DIASTOLIC BLOOD PRESSURES AT VARIOUS RATES OF ADRENALIN ADMINISTRATION

DOSE OF ADRENALIN ( $\mu\text{C/KG/MIN}$ )	CORRELATION COEFFICIENT
0	0.817
0.11 0.30	0.754
0.21 0.50	0.795
0.51 0.70	0.781
0.71 0.90	0.814
0.91 1.10	0.718
1.11 1.30	0.885
1.31 1.50	0.529
Average	0.800

The perfusion ratio averaged  $1.7 \pm 0.27$  and did not vary materially among the four pressure groups. The size of this ratio was somewhat surprising for its value at normal pressures is usually stated to approximate 1.0<sup>12, 23b</sup> while the increased peripheral resistance in hypertensive subjects is expected to exaggerate the diastolic pressure<sup>4</sup> and yield a ratio lower than normal. However, a calculation of this ratio from data reported by Steele<sup>4</sup> for a group of thirty-nine patients whose blood pressures ranged from 94 mm Hg systolic, 60 mm diastolic to 262 mm systolic, 164 mm diastolic gave an average value of  $1.72 \pm 0.10$ . Similar calculations were made from Golding and

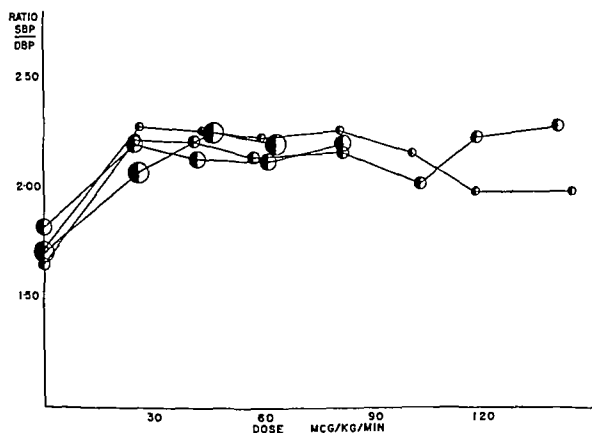


Fig. 6—Average change in systolic diastolic ratio produced by adrenalin in subject groups on the basis of initial diastolic pressure. Size of circles indicate the relative magnitude of the initial blood pressure range.

Chasis data<sup>15 pp 20 21</sup> relative to fifty-six patients with mild to severe hypertension. The ratio of this latter group averaged  $1.59 \pm 0.19$  for both minimum and maximum recorded pressures.

At the lowest rate of adrenalin administration, the systolic-diastolic ratio in the present series increased to an average value of  $2.21 \pm 0.38$ . This new value was also similar in all pressure groups and tended to remain constant with succeeding increments in dosage. These relations are illustrated in Fig 6 and their variability indicated in Table V. The *t* value (9.78) for the difference in ratio before and during infusion at the lowest dose range indicated the change to be highly significant.

TABLE V SYSTOLIC-DIASTOLIC PRESSURE RATIO IN RELATION TO INITIAL DIASTOLIC BLOOD PRESSURE, VARIOUS RATES OF ADRENALIN ADMINISTRATION

INITIAL DBP RANGE	RANGE OF ADRENALIN DOSAGE ( $\mu\text{G/KG/MIN}$ )								<i>c</i>
	0	0.11-0.30	0.31-0.50	0.51-0.70	0.71-0.90	0.91-1.10	1.11-1.30	1.31-1	
51-70	$1.72 \pm 0.21$	$2.28 \pm 0.25$	$2.26 \pm 0.31$	$2.23 \pm 0.28$	$2.26 \pm 0.31$	$2.16 \pm 0.25$	$1.98 \pm 0.32$	$1.98 \pm 0$	
71-90	$1.75 \pm 0.26$	$2.22 \pm 0.53$	$2.21 \pm 0.47$	$2.14 \pm 0.38$	$2.16 \pm 0.13$	$2.02 \pm 0.50$	$2.23 \pm 0.15$	$2.25 \pm 0$	
91-110	$1.72 \pm 0.34$	$2.20 \pm 0.35$	$2.13 \pm 0.38$	$2.12 \pm 0.30$	$2.20 \pm 0.39$				
111-130	$1.71 \pm 0.07$	$2.07 \pm 0.07$	$2.25 \pm 0.13$	$2.20 \pm 0.12$					
Average	$1.73 \pm 0.27$	$2.21 \pm 0.38$	$2.21 \pm 0.37$	$2.17 \pm 0.31$	$2.22 \pm 0.26$	$2.10 \pm 0.30$	$2.12 \pm 0.24$	$2.10 \pm 0$	

It would appear likely that this increase in systolic-diastolic ratio reflects a readjustment in the relation of peripheral resistance to cardiac output which results from adrenalin action and is relatively independent of the initial pressure.

*Postinfusion Fall in Pressure*—When blood pressure is raised by application of a pressor agent which can be quickly destroyed, it is anticipated that the pressure will return at least to the initial level when the pressor agent is no longer supplied. In order to avoid such spurious correlations, all calculations involving the fall which followed adrenalin infusion were based on the amount by which the blood pressure dropped below the preinfusion level.

Computed on this basis, the postinfusion fall in both systolic and diastolic blood pressures was found to correlate more closely with the height of the initial blood pressure than with rise in pressure, maximum pressure, dose or maximum unit change in blood pressure, as shown in Table VI.

TABLE VI CORRELATION BETWEEN FALL IN POSTINFUSION BLOOD PRESSURE BELOW INITIAL LEVEL AND VARIOUS MODIFYING INFLUENCES

FALL IN BLOOD PRESSURE AND	COEFFICIENT OF CORRELATION (SYSTOLIC PRESSURE)	COEFFICIENT OF CORRELATION (DIASTOLIC PRESSURE)
Initial pressure	-0.598	-0.491
Maximum pressure	-0.500	-0.307
Maximum change in pressure	0.032	0.134
Dose	-0.389	-0.117
Change in unit pressure	0.079	0.230

In general, the higher the patient's initial pressure, the further did the pressure drop below the initial level when the infusion was stopped. The extent of this relation is illustrated in Figs 7 and 8. In a number of instances the

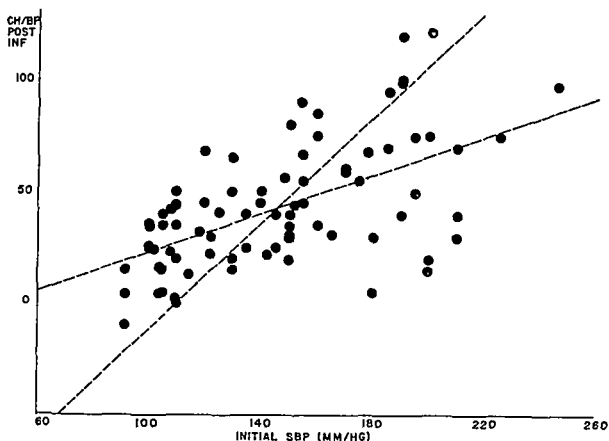


Fig 7—The relation between initial systolic pressure and the postinfusion fall below initial pressure. The lines of regression from the X and Y axes are indicated

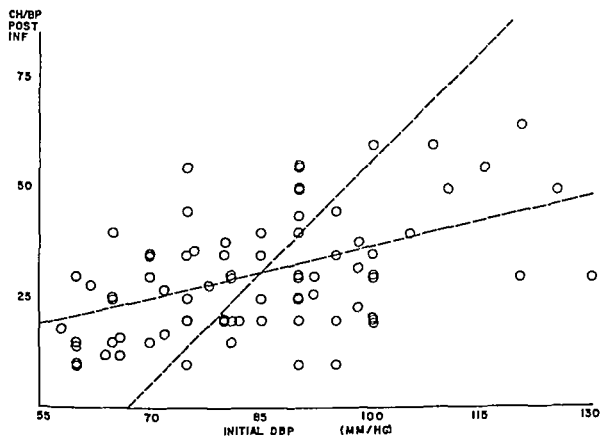


Fig 8—The relation between initial diastolic pressure and the postinfusion fall below initial pressure. The lines of regression from the X and Y axes are indicated

minimum pressures came within the range usually associated with shock. Except for tachycardia, however, no other signs of shock were noted. The subjects remarked about a sensation of heat and manifested a widespread vasodilatation by a generalized flushing of the skin. Tilting during the stage of blood pres

sure depression was followed by considerable acceleration of the pulse rate and in one instance by syncope. The reactions to tilting were similar to the ones which follow bleeding.<sup>26</sup>

The cause of the postinfusion drop in tension to subnormal levels is questionable. The depression of pressure in association with evidences of vasodilatation suggests a temporary persistence of compensatory vasodepressor activity, either nervous or humoral. The presence of postural vasomotor reflexes makes it unlikely that adrenalin blockage of sympathetic ganglia<sup>27</sup> is the responsible factor. The tendency of the hypertensive subjects to display a more marked postinfusion pressure depression accords with the results of carotid sinus stimulation.<sup>1</sup>

The possible role of blood volume changes is considered in connection with blood concentration.

*Alterations in Hemococentration*—Two potential mechanisms exist by which adrenalin might alter blood concentrations. The first involves splenic contraction followed by extrusion of sequestered red cells and an increase in their percentage in the circulating blood.<sup>23a, 24, 28</sup> The second possibility implicates leakage of plasma through capillaries made more permeable by stagnant anoxia following prolonged vasoconstriction.<sup>29</sup>

These possibilities were studied by using the hematocrit value as an indicator of blood concentration. The results of determinations made during and after infusion were compared with the initial values. No significant changes were demonstrated at either stage within the range of dosage used (Table VII).

The constancy of the hematocrit value makes it unlikely that the postinfusion blood pressure depression displayed by the subjects of this investigation was due to a reduction in plasma volume comparable to that produced in dogs by Freeman and co-workers through administration of adrenalin in shock dosages.<sup>30</sup>

TABLE VII EFFECTS OF ADRENALIN INFUSION ON BLOOD CONCENTRATION

TIME	HEMATOCRIT VALUE (VOL %)
a Average Values	
(1) Before infusion	39
(2) During infusion	40
(3) After infusion	39
b Standard Deviations of the Differences in Mean Hematocrit Value During and After Infusion as Compared with Preinfusion Average	
d (1) and (2)	$1 \pm 2.9$
d (1) and (3)	$0 \pm 3.7$

#### SUMMARY AND CONCLUSIONS

Administration of eighty continuous infusions of adrenalin to fifty-one subjects evoked a common pattern of systolic and diastolic response of all ranges of initial pressure.

The correlation between the height of the initial pressure and the depth of the subsequent postinfusion depression provides additional data that the vasodilation capacity of the hypertensive individual is enhanced rather than diminished.

The sum of these responses to adrenalin together with the extent of the fall produced by carotid sinus stimulation spinal anesthesia pyrogens and the tetraethylammonium ion indicates that 'fixed hypertension' in the sense of a pressure elevation incapable of material reduction is a concept of questionable reality. On the contrary the accumulated evidence demonstrates the availability of compensatory mechanisms in the hypertensive subject and the capacity of the vascular apparatus to respond effectively when these mechanisms are properly stimulated.

The existence of pressure elevation in the presence of potentially effective restorative mechanisms suggests that sustained hypertension is associated like fever, with an upward shift in the base line from which the homeostatic mechanisms are operative. The problem then is to determine the factor or factors responsible for the elevation of the pressure base line to this new level.

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# THE EFFECT OF OPERATION AND ILLNESS ON CLOT RETRACTION DESCRIPTION OF A NEW METHOD

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LAMPERT,<sup>1</sup> in 1935, indicated the possible role which accelerated clot retraction may play in phlebothrombosis and pulmonary embolism. Hirschboeck and Coffey have recently demonstrated that a shortening of the clot retraction time occurs in patients who have had a recent pulmonary embolism. Because of the difficulty in observing the end point in the usual blood coagulation and clot retracting tests, it was decided to search for a more simple and decisive method of observation. The coagulation retraction test herein described has been found to be more useful than the older methods and was used in obtaining the data for this report.

Clot retraction is dependent upon thrombocytes, surface forces, erythrocyte mass, and qualitative and quantitative variations in fibrin. Other factors, as yet unknown, also may influence the process. It is common knowledge that clot retraction is greatly delayed or does not occur when thrombocytopenia is present. Thrombocytosis is, on the other hand, associated with rapid clot retraction.<sup>2</sup>

It is not known how the thrombocytes actually participate in the phenomenon. Tocantins' explanation that the agglutinated, disintegrating thrombocytes which collect and adhere to the fibrin strands fuse with adjacent thrombocyte masses thus causing the gradual pulling together of the fibrin mesh, has been widely accepted.<sup>3</sup>

The surface with which the clot is in contact greatly influences the retraction. A collodion surface is completely inhibitory.<sup>4</sup> Paraffin, methyl methacrylate, and other water repellent materials which inhibit coagulation also more or less inhibit clot retraction. The surface of the vascular endothelium apparently exerts a similar effect. These surfaces may act because of their ability to inhibit platelet agglutination or because the fibrin becomes more firmly attached to the surface.<sup>5</sup>

Erythrocytes influence clot retraction in two ways. First, when the concentration as measured by the hematocrit is abnormally great, for example 80 per cent (a value frequently seen in congenital heart disease with chronic anoxemia), clot retraction does not occur. The mass of erythrocytes which incidentally, has an entirely passive role in coagulation, limits the degree of retraction by the volume which it occupies in the clot. Second, when the erythrocyte mass is large, the plasma volume and hence the total amount of fibrinogen, is proportionately small. The opposite condition prevails in anemia. Here the small erythrocyte mass interferes less with clot retraction and the clot

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Fig. 1.—The effect of surface on platelet disintegration and fibrin formation. The upper photograph was taken one hour after plasma from freshly drawn centrifuged human blood was placed on a glass surface (microscope slide and cover slip). Note the disintegrating platelets and the numerous delicate fibrin strands. The large bodies are erythrocytes. The lower photograph demonstrates the effect of a collodion surface on the same specimen of plasma. The platelets are intact and highly refractile and the fibrin strands are heavier and more numerous.

contains more fibrin because of the relative or even absolute hyperfibrinogenemia. Thus anemic blood exhibits a tendency toward more rapid and extensive clot retraction. A fibrinogen fraction which greatly accelerates erythrocyte sedimentation and is converted into rapidly retracting fibrin (contractinogen)<sup>6</sup> may be present. These qualitative and quantitative increases in fibrin which occur during illness are responsible not only for increasing the rate of erythrocyte sedimentation but also for producing denser and more retractile clots.

A study of the thrombotic diathesis or, as some prefer to say, hypercoagulability, must include more than the mere measurement of coagulation time. A method which will measure indirectly the combined effect of anemia, thrombocytosis, and hyperfibrinogenemia in addition to any other factor which may express itself in a shortened coagulation time, should be valuable. Earlier observations of the clot retraction time form the basis for much of the present work. A new method using capillary blood has been devised. It has the advantages of eliminating venipuncture and of having a more precise end point.

#### METHOD FOR DETERMINING THE CLOT RETRACTION TIME IN CASTOR OIL

The skin of the finger tip is cleansed with alcohol and a puncture 3 mm deep is made with a No. 11 Bard Parker blade with a coil guard set 3 mm from the tip. The time is recorded when the skin puncture is made. Two samples of blood (20 cmm each) are drawn as quickly as possible into Sahli hemoglobinometer pipettes. The same pipette may be used for each sample if the blood flows freely and the aspiration is done quickly. Each sample of blood is suspended in a separate test tube filled with castor oil U. S. P. in the following manner. The blood is expelled from the pipette as a large single drop which is planted on the center of the oil surface by touching the tip of the pipette to the oil. The drop will settle into the oil and hang by surface attraction to the meniscus. Castor oil was selected because its specific gravity at room temperature is almost the same as the specific gravity of blood. The tubes are stoppered to prevent evaporation. This procedure should not take longer than twenty seconds. Two samples are used for purposes of control. If the clot retraction time is not equal or within three minutes of being equal in both tubes, the test is repeated. This is seldom necessary. The tubes are placed in a rack at room temperature and are observed at the end of ten, fifteen, and twenty minute periods. The end point of coagulation is not determined. The beginning of clot retraction is established when a visible dimpling of the clot surface with extrusion of a tiny droplet of serum occurs. It is usually readily seen because of the highly retractile oil blood interface. This is the end point of the test. The droplet of serum enlarges until clot retraction is complete (Figs. 2 and 3). For investigative purposes, the preparations were observed continually.

It was found that normal individuals have a clot retraction time of 20 minutes or longer, the average being 33.1 minutes. Clot retraction times longer than 50 minutes were observed only in thrombocytopenia and erythremia and in persons with a coagulation defect. The clot retraction time may fluctuate during the course of the day in some individuals. The moving of patients in





Fig 3—Three types of serum buds observed when a drop of capillary blood coagulates and retracts in capillary oil

and out of bed has been associated with fluctuations of more than 8 minutes in some of the more senile individuals. It is thought that this may be due to minor variations in thrombocyte levels. The clot retraction time is shorter at  $37^{\circ}\text{C}$ . Variations in room temperature have not appreciably influenced the results. Room temperature was selected for the test because the drops of blood frequently fall in the oil at  $37^{\circ}\text{C}$ .

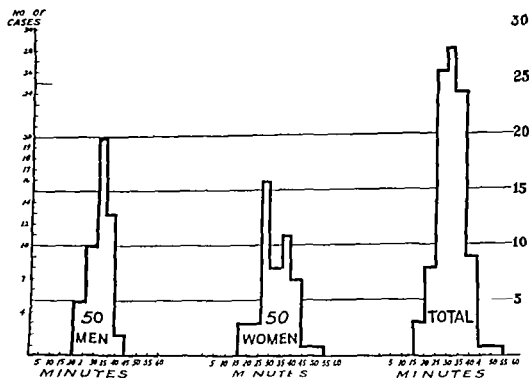


Fig 4—Normal variations in the coagulation retraction time in a series of one hundred normal individuals

The clot retraction time was determined in a series of fifty normal men and fifty normal women. Hospital attendants, nurses, and medical students were used as subjects. The mean clot retraction time for men was 32.4 minutes and for women was 33.8 minutes, or 33.1 minutes for the group as a whole (Fig 4). It was noticed that active menstruation slightly shortened the clot retraction time. Eleven women had a mean clot retraction time of 27.6 minutes during active menstruation and 34.7 minutes during the intermenstrual period.

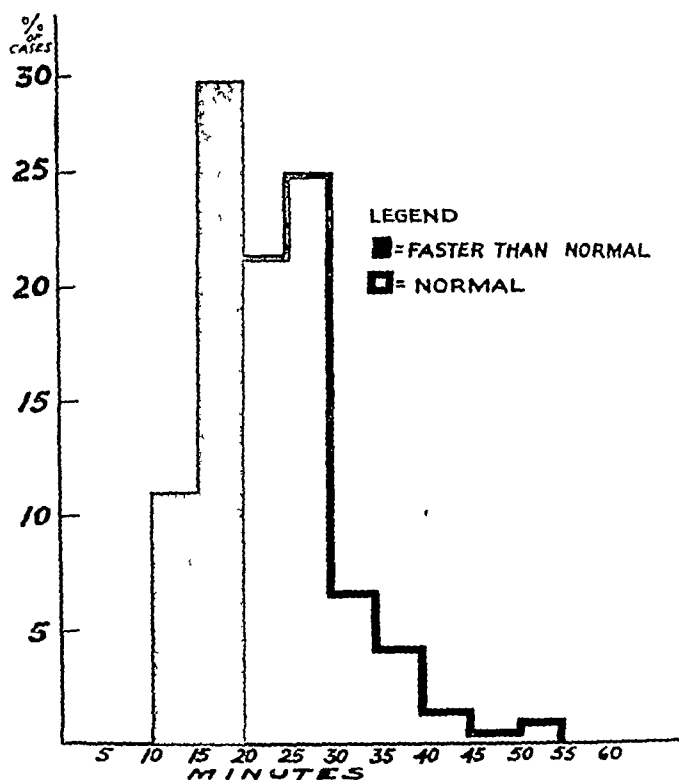


Fig 5—The shift to the left in coagulation-retraction times observed in three hundred general medical patients

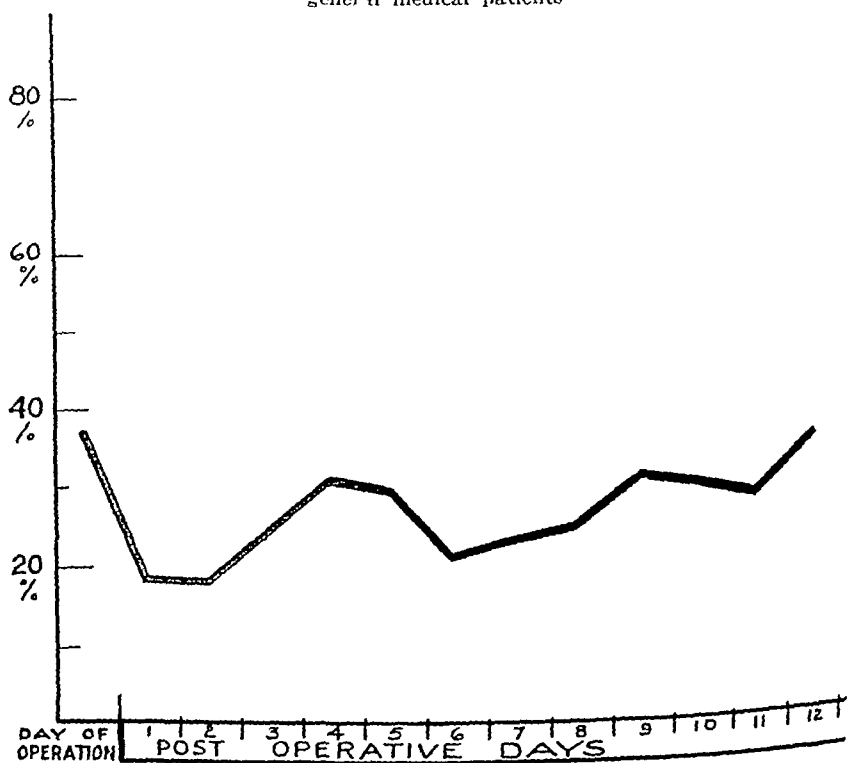


Fig 6—Postoperative variations in the incidence of short (pathologic 20 minutes or less) coagulation-retraction times

Observations in the general hospital patient population, excluding patients in the first postoperative month revealed that the mean clot retraction time was shorter than in healthy persons (Fig 5). The mean clot retraction time for one hundred fifty two men was 24.9 minutes and for one hundred forty eight women, 22.6 minutes, or 23.8 minutes for the total group (three hundred). The clot retraction time was less than 20 minutes in 23.7 per cent and less than 15 minutes in 7.6 per cent of the group.

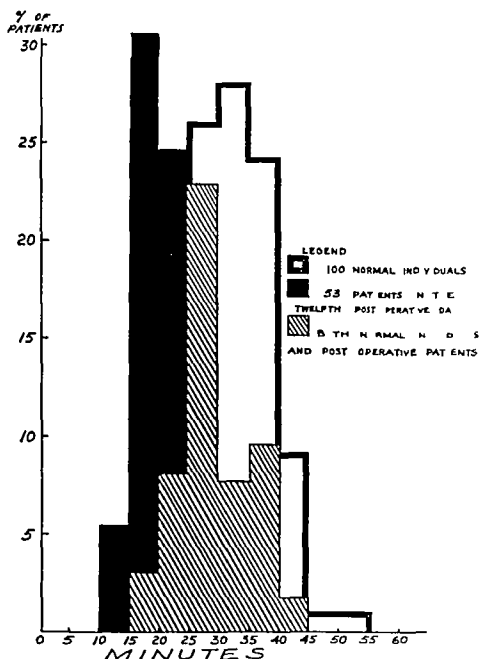


Fig 7—A comparison between the coagulation retraction times of normal and postoperative patients. The cross ruled area represents the distribution region common to both groups.

One hundred surgical patients were observed during the postoperative period. The majority had undergone major abdominal surgery. Fig 6 shows the tendency of the clot retraction time to fluctuate during the postoperative period. Of the tests performed on the day of surgery, 33.5 per cent were faster than normal. This may be explained by the hyperfibrinogenemia resulting from the stimulus of surgical trauma and perhaps dehydration. The percentage of abnormally short clot retraction times was also high on the third, fourth, and fifth and on the ninth and subsequent postoperative days. Fig 7 is a comparison between the clot retraction time of fifty three patients in their twelfth post

operative day with one hundred normal individuals. Seventy six of the one hundred postoperative patients had an abnormally short clot retraction time on one or more days during the period. In thirty of the patients the clot retraction time was shorter than 15 minutes on one or more days.

#### DISCUSSION

In the past, great emphasis has been placed on vascular endothelial trauma and delayed circulation time as factors in the cause of phlebothrombosis and pulmonary embolism. Since the introduction of anticoagulant therapy more attention has been given to the blood itself. The thrombotic diathesis apart from slowing of circulation time and vascular trauma, is entirely dependent upon hemie factors, namely thrombocytosis, hyperfibrinogenemia, and diminished erythrocyte mass. These factors may enhance coagulation, but their more important effect lies in their creation of a strongly retracting, tough clot which is susceptible to embolic detachment because of its retractility.

One of the patients in the nonsurgical group, a woman who had recently been digitalized for early total myocardial failure, developed pulmonary embolism when her clot retraction time was 11 minutes. The clinical picture was typical and the radiographic examination of the chest and the electrocardiogram sustained the diagnosis. This was the only instance of pulmonary embolism encountered during the entire study. A correlation between the clot retraction time in castor oil and the tendency toward pulmonary embolism is implied in this case. Likewise the shortening of the clot retraction time in bedridden patients may help to explain the tendency toward pulmonary embolism prevalent in that group. If we can assume that patients with short clot retraction times are more susceptible to thrombosis and embolism, it may be desirable to treat those persons prophylactically with anticoagulants.

The clot retraction test is well adapted to the control of heparin therapy and can be used instead of the ordinary coagulation time determinations. It is not reliable during Dicumarol therapy. The test is really a measure of the coagulation time plus the interval between the end of coagulation and the beginning of clot retraction. Heparin since it prolongs coagulation and in some instances inhibits the postcoagulation phase, will greatly lengthen the time of the test. Dicumarolization frequently prolongs the clot retraction time, but there is no correlation between this effect and the prothrombin time.

#### SUMMARY

A test for measuring the blood clot retraction time in castor oil is described.

The clot retraction time is considerably shorter than normal in certain patients who are in the postoperative period and in almost one fourth of all general medical patients.

The test measures factors which are part of the hemie phase of the thrombotic diathesis.

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## LABORATORY METHODS

### A SIMPLIFIED TECHNIQUE FOR THE QUANTITATIVE COLORIMETRIC ESTIMATION OF PREGNANDIOL IN URINE

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PREVIOUSLY this laboratory reported a simple method for the qualitative determination of urinary pregnandiol by its reaction with concentrated sulfuric acid<sup>1, 2</sup>. The present report deals with the study of the pregnandiol color reaction which permits its simple quantitative measurement in a colorimeter.

#### REAGENTS

Toluene, chemically pure

Concentrated hydrochloric acid, chemically pure

0.1 N sodium hydroxide

Two per cent sodium hydroxide in absolute methanol. A 4 to 8 per cent solution of sodium hydroxide in absolute methanol is first prepared by adding sodium hydroxide pellets to an Erlenmeyer flask containing absolute methanol. The mixture is filtered through a dry fritted glass filter to remove the precipitated carbonate. The sodium hydroxide concentration of the filtrate is determined by titration with 0.1 N sulfuric acid. The filtrate is then adjusted to a concentration of 2 per cent with absolute methanol. The solution is freshly prepared every week.

Acetone, chemically pure

Absolute ethyl alcohol

Concentrated sulfuric acid, chemically pure

#### TECHNIQUE\*

##### *A. Hydrolysis and Excretion of Pregnanediol —*

1. One hundred milliliters urine, 50 ml. toluene, 10 ml. concentrated hydrochloric acid, and two glass beads are added to a 500 ml. flat-bottomed Florence flask.

Department of Metabolism and Endocrinology, Research Institute, Michael Reese Hospital. The department is in part supported by the Michael Reese Research Foundation. This work was aided by a grant from the Committee on Scientific Research of the American Medical Association to Dr. Rachmiel Levine.

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\*Based upon the method for pregnandiol extraction by Astwood and Jones<sup>3</sup> and the color reaction of Talbot and co-workers.<sup>4</sup>

2 The flask is connected via a one holed cork stopper to a Liebig condenser (water-cooled 400 to 500 mm jacket length) in vertical position and the mixture is boiled vigorously over an electric hot plate for fifteen minutes

3 The flask and its contents are brought to room temperature by cooling under the water tap

4 The mixture is transferred to a 500 ml separator funnel and the lower layer (urine) is drawn off

5 The toluene layer and emulsion are washed twice with 15 ml portions of 0.1 N sodium hydroxide and then twice with 15 ml portions of distilled water

#### *B Precipitation of Impurities —*

1 The washed toluene and emulsion (A 5) are transferred to a 125 ml Erlenmeyer flask with two glass beads

2 The mixture is boiled over an electric hot plate (in the hood)

3 When the water has evaporated and the toluene mixture is boiling smoothly 10 ml of 2 per cent sodium hydroxide in absolute methanol are added

4 The mixture is evaporated until a granular precipitate appears and approximately one half of the original toluene volume is reached

5 The toluene mixture is then filtered while hot through a fritted glass filter (medium porosity Pyrex) with mild suction (If the filtrate has an orange pink or brown tinge steps B 3, B 4 and B 5 must be repeated until the filtrate is yellow or yellow green)

6 The precipitate (B, 5) is washed with 15 ml hot toluene

7 The combined filtrates (B 5 and B 6) are then evaporated to dryness over the hot plate (in the hood) a gentle air stream being used to drive off the last traces of toluene This avoids charring of the residue

#### *C Precipitation of Pregnandiol —*

1 Five milliliters acetone are added to the residue (B 7) and the mixture is warmed over a hot plate until solution is complete

2 Twenty milliliters 0.1 N sodium hydroxide are added slowly and the mixture is boiled for three minutes on the hot plate

3 The flask is then placed in a refrigerator (5° C) for one hour

#### *D Isolation of Pregnandiol —*

1 The mixture (C 3) is filtered through a fritted glass filter (medium porosity Pyrex) with mild suction

2 The precipitate (D, 1) is washed with 15 ml distilled water

3 The receiving flask is changed and 10 ml hot absolute alcohol are passed through the fritted glass filter to dissolve the precipitate

4 The alcohol filtrate (D 3) is evaporated to dryness from the receiving flask over an electric hot plate (in the hood)

#### *E Color Development and Quantitative Measurement —*

1 Ten milliliters concentrated sulfuric acid are added to the residue (D 4)

2 Color is allowed to develop for one hour

3 An aliquot of the solution E, 2 is diluted up to a final volume of 5 ml with concentrated sulfuric acid in a dry absorption tube (Cenco No 1234M tubular cell) and the solution is thoroughly mixed

4 The color is read on a spectrophotometer\* at 430  $m\mu$  (concentrated sulfuric acid is used as the blank)

5 The amount of pregnandiol represented by the absorption reading is obtained from the standardization curve. Calculations for the dilutions involved are made to ascertain the pregnandiol present in the original sample

#### EXPERIMENTAL

The evidence that pregnandiol is the steroid extracted by this procedure is based on the spectrophotometric absorption and the melting point data which follow

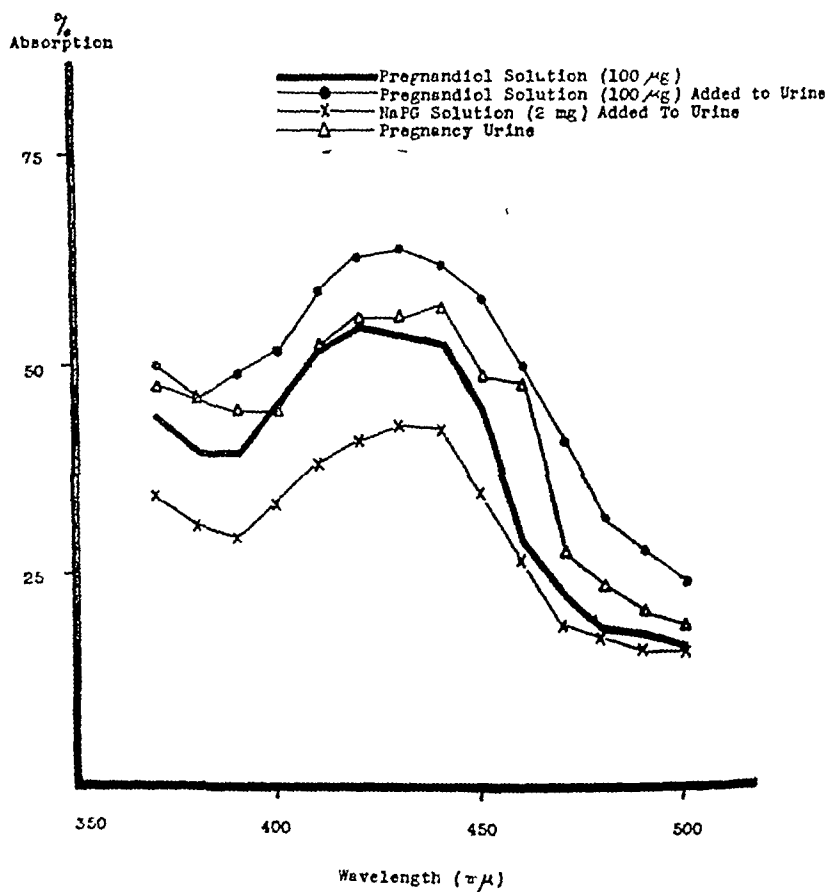


Fig 1—Absorption curves of color complexes obtained from the addition of concentrated sulfuric acid to crystalline pregnandiol and pregnandiol recovered from urine

Pregnancy urine pooled female urine to which sodium pregnandiol glucuronide (NaPG)<sup>†</sup> was added, and pooled female urine to which pregnandiol was added were subjected to the foregoing procedure. The absorption spectra of the color complexes obtained with these specimens and the absorption spectra

\*The Cenco-Sheard Spectrophotometer Central Scientific Company Chicago Ill was used in the present study

<sup>†</sup>Sodium pregnandiol glucuronide will be designated as NaPG in the succeeding paragraphs

trum of crystalline pregnandiol to which concentrated sulfuric acid had been added were compared. Fig 1 illustrates the four curves. They resemble one another closely and demonstrate an absorption maximum between 420 and 440 millimicrons.

The melting points of the products recovered in this experiment are listed below.

- 1 Pregnancy urine, 205 to 215° C
- 2 NaPG added to pooled female urine 215 to 227° C

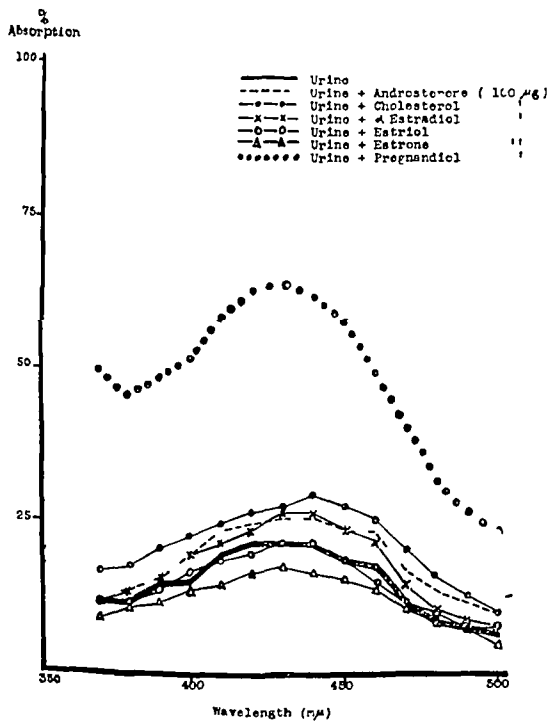


Fig 1—Absorption curves of the products recovered from urine to which various steroids had been added.

- 3 Pregnanediol added to pooled female urine, 227 to 233° C
- 4 Crystalline pregnandiol, 234.5 to 237° C

When the recovered substances (from 1, 2, and 3) were each mixed with pure pregnandiol there was no depression of the melting point.

The absorption curves of other steroids commonly found in urine were studied in similar fashion. Androstosterone, cholesterol,  $\alpha$ -estradiol, estriol, estrone and pregnandiol,\* 100  $\mu$ g of each in toluene solution, were added to 100 cc samples of pooled female urine and the mixture was run through the recommended procedure. Fig 2 illustrates the absorption curves obtained. It appears from these data that the steroids tested do not significantly alter the absorption curve (at the given wave lengths) of the pooled female urine used as a control. Only the specimen with added pregnandiol demonstrated a marked increase in absorption with a maximum at 430 millimicrons.

These data appeared to indicate that the method outlined leads to the recovery of pregnandiol alone.

Optimal conditions for the quantitative extraction of pregnandiol from urine and the maximal final color development were established by the following experiments.

*I Duration of Simultaneous Hydrolysis and Extraction*—NaPG (aqueous solution) 2 mg, was added to 100 ml portions of pooled female urine†. The specimens were refluxed for five, fifteen, and thirty minutes each and then carried through the procedure. The results obtained indicated that maximal extraction of pregnandiol occurs if the urine mixture is refluxed for fifteen minutes. Five minutes of boiling appeared inadequate for optimal recovery of pregnandiol. Refluxing for thirty minutes did not interfere with the maximal recovery. It was therefore concluded that specimens should be refluxed for at least fifteen minutes.

*II Efficiency of One Period of Hydrolysis and Extraction*—Toluene 50 ml was added to the urine layers of the specimens (I) which had been hydrolyzed and extracted for fifteen minutes. Some of the samples were extracted in the cold for fifteen minutes on a mechanical shaker. The remainder were refluxed for an additional fifteen minutes as in the original procedure. The toluene layers were saved and carried through the technique. The absence of color development at step E, 2 indicated that in the first period of boiling the extraction of urinary pregnandiol was complete.

### *III The Effect of Aqueous Sodium Hydroxide*—

A A series of specimens were carried to step A 5. At that point the toluene layers were each washed with one, two, and four 15 ml portions of 0.1 N sodium hydroxide. The technique was then followed as outlined. The results of this experiment showed that at least two washings of the toluene layer with 0.1 N sodium hydroxide are necessary for uniform neutralization. Additional washings with 0.1 N sodium hydroxide did not affect pregnandiol recovery.

B The combined sodium hydroxide and water washings of the specimen (III A) were diluted to a volume of 100 ml with distilled water. Ten milli-

\*The androstosterone,  $\alpha$ -estradiol, estriol, and estrone were supplied through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J. The pregnandiol and sodium pregnandiol glucuronide were generously supplied by Ayerst McKenna and Harrison Ltd., Montreal, Canada.

†Unless otherwise indicated in the following experiments 2 mg sodium pregnandiol glucuronide were added to 100 ml portions of pooled female urine. All tests were carried out in duplicate.

liters hydrochloric acid (concentrated) and 50 ml of toluene were added. These mixtures were carried through the procedure (starting at A 1). No color development occurred in any specimen. Thus, it appeared that the sodium hydroxide originally used did not remove pregnandiol from the toluene layer.

#### *IV Precipitation of Impurities by 2 Per Cent Sodium Hydroxide in Absolute Methanol —*

A One hundred milliliter portions of pooled female urine to which no NaPC was added were carried to step B 3. At that point, 0.5, 10, 20 and 30 ml aliquots of 2 per cent sodium hydroxide in absolute methanol were added and the procedure was completed. When the methanolic sodium hydroxide solution was not employed, the red and violet urinary pigments extracted by the toluene were not removed from solution and they interfered with the final color reaction by producing bizarre colors. The use of sodium hydroxide in methyl alcohol removed these urinary pigments and resulted in constant final color development. Therefore it was concluded that methanolic sodium hydroxide should be employed at this stage.

B When the same experiment was carried out with urine specimens to which NaPG had been added the following observations were made. When 5 ml of sodium hydroxide in methanol were used the color reaction developed represented more than 100 per cent recovery of pregnandiol.

Since the control urines (IV A) indicated that methanolic sodium hydroxide is necessary to remove interfering substances it appeared that 5 ml of sodium hydroxide in methanol might have been inadequate to remove all the nonpregnandiol chromogens. The addition of 10 ml of sodium hydroxide in methanol resulted in pregnandiol recoveries of over 90 per cent. The use of 20 to 30 ml of sodium hydroxide in methanol at step B 3 reduced the pregnandiol recovery. It was possible to recover pregnandiol from the sodium hydroxide precipitates (at step B, 5) of the specimens in which 20 and 30 ml of methanolic sodium hydroxide had been used. It was concluded therefore that 10 ml of 2 per cent sodium hydroxide in absolute methanol would be adequate to remove interfering substances without precipitating pregnandiol prematurely.

1 *Period of Precipitation*—A series of specimens was carried to step C 1. In the original communication<sup>1</sup> it was suggested that aqueous 0.1 N sodium hydroxide be added to the warmed acetone solution to precipitate the pregnandiol. Our quantitative studies indicated that under such conditions the precipitation of pregnandiol reached a maximum after three hours of refrigeration at 5° C. However, it was found that if the sodium hydroxide acetone mixture were boiled three minutes the maximal pregnandiol precipitation occurred in one hour. Extension of the boiling period led to the precipitation of nonpregnandiol chromogens which interfered with the final color reaction.

VI *Color Development With Sulfuric Acid*—The color reactions developed by solutions containing various amounts of crystalline pregnandiol (8.40 and 50 µg) were examined on the colorimeter at frequent intervals starting ten minutes after the addition of the acid. Maximal absorption at 430 mµ occurred

between thirty and sixty minutes later. Since the more concentrated specimens reached their maximal absorption with one hour of development, we employed this standard time interval for reading all our specimens on the spectrophotometer.

**VII Standardization Curves**—Aliquots of a solution of pregnandiol in absolute alcohol (100  $\mu\text{g}$  per cubic centimeter) were measured from a microburette into test tubes. The alcohol was evaporated from the tubes in a glycerine bath. After cooling, 5 ml of sulfuric acid (concentrated) were buretted into each tube. The color was allowed to develop for one hour (the tubes being shaken occasionally) and was read on the spectrophotometer at 430  $m\mu$  with concentrated sulfuric acid used as a blank. Fig 3 represents the average results obtained. In the range of 10 to 100  $\mu\text{g}$  per unit volume (5 ml), the concentration is proportional to the log of the per cent absorption, obeying Beer's law. With concentrations greater than 100  $\mu\text{g}$  this relationship does not obtain. A similar standardization curve was obtained when pregnandiol was recovered from pooled female urine to which NaPG had been added in varying amounts.

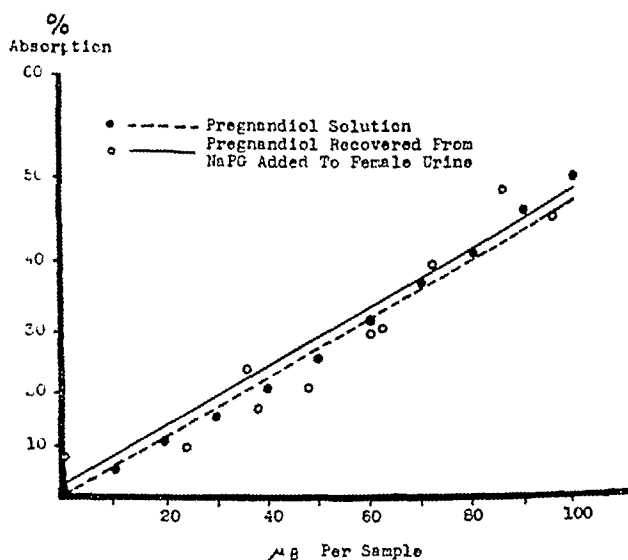


Fig 3—Standardization curves of the color reaction (with sulfuric acid) of crystalline pregnandiol and pregnandiol recovered from female urine to which NaPG had been added.

The curve is also illustrated in Fig 3. Each point represents the average value of five to ten results. This curve parallels that of pure pregnandiol and indicates that the amount of pregnandiol recovered varies directly with the NaPG present in urine.

To determine the efficiency of the method in recovering pregnandiol from NaPG added to urine, the following calculations were considered:

- 1 Sodium pregnanolone glucuronide constitutes 20 per cent of NaPG.
- 2 The molecular weight of pregnandiol is 320. The molecular weight of pure sodium pregnandiol glucuronide is 536.

Therefore the pregnandiol theoretically obtainable from sodium pregnandiol glucuronide can be calculated from the following equation (where A = weight of NaPG [in mg])

$$\frac{MW \text{ (pregnandiol)}}{MW \text{ (NaPG)}} \times (A - 0.2A) = \text{weight of pregnandiol (in mg)}$$

Table I represents recoveries of pregnandiol from NaPG calculated as indicated. It will be seen that an average of 95 per cent of the theoretic amount available was recovered.

TABLE I RECOVERY OF PREGNANDIOL FROM SODIUM PREGNANDIOL GLUCURONIDE

NAPG ADDED (MG)	CALCULATED EQUIVALENT OF PREGNANDIOL (MG)	PREGNANDIOL RECOVERY	
		(MG)	(%)
0.50	0.24	0.17	71
0.75	0.36	0.42	117
0.80	0.38	0.30	79
1.00	0.48	0.37	77
1.25	0.60	0.55	92
1.30	0.62	0.57	92
1.50	0.72	0.78	108
1.90	0.86	1.04	120
2.00	0.96	0.95	99
		Average	95

## DISCUSSION

Methods for the quantitative determination of urinary pregnandiol either as the conjugated form, sodium pregnandiol glucuronide or as free pregnandiol have been reported previously.<sup>3, 4, 7, 10</sup> The majority of the methods require large volumes of urine, consume several days in performance, and usually depend on a gravimetric technique for measuring the final product. The technique reported at this time requires small urine volumes and consists of technically simple manipulations. One person can perform twelve determinations in a working day. The equipment is standard for chemical laboratories and no special apparatus or chemicals are required. The quantitative estimation is a colorimetric procedure which can be adapted to simple laboratory colorimeters. Although the present report considers results obtained with the Cenco Sheard spectrophotometer, equally satisfactory results have been obtained in this laboratory with the simpler Klett Summerson photoelectric colorimeter (using Filter 42).

In carrying out this procedure technical difficulties may be prevented if precautions discussed elsewhere are observed.<sup>2</sup> These simple measures consist of (1) the use of chemically pure reagents, (2) the preparation of fresh 2 per cent sodium hydroxide in absolute methanol every week, (3) the use of air stream in preventing charring of residues at steps B 7 and D 4 and (4) the use of fritted glass filters.

Emulsions have been encountered infrequently at step A, 5. Urine specimens containing large amounts of albumin and/or blood contribute to such emulsion formation. Filtering the visibly bloody specimens reduces the inci-



dence of emulsions. The addition of 1 to 2 drops of a detergent to the toluene mixture usually breaks up the emulsion and facilitates the procedure.

If it is necessary to interrupt the procedure before completion this may be done conveniently at steps B, 2, B, 7 and D, 4.

The observations reported in this paper lead us to conclude that the method proposed permits the determination of pregnandiol alone. Androstosterone (17 ketosteroids) was not found to interfere with the color reaction. The quantitative estimation of pregnandiol in a given urine specimen depends on its spectrophotometric absorption (at  $430\text{ m}\mu$ ). This reading is compared with the standard curve which is a representation of the actual amount of pregnandiol recoverable from urine to which sodium pregnandiol glucuronide has been added.

Failure to use pregnandiol as a standard or to read the color absorption at  $430\text{ m}\mu$  has led to results which appear to us to have been interpreted erroneously.<sup>11, 12</sup> Reinhardt and Barnes<sup>12</sup> utilized 2 per cent potassium dichromate in water (at  $420\text{ m}\mu$ ) as a standard for measuring pregnandiol. Since this solution is fifty times more concentrated than the amount of potassium dichromate equivalent to the pregnandiol standard previously recommended by this laboratory (0.4 mg pregnandiol)<sup>2</sup> it is not surprising that their results were

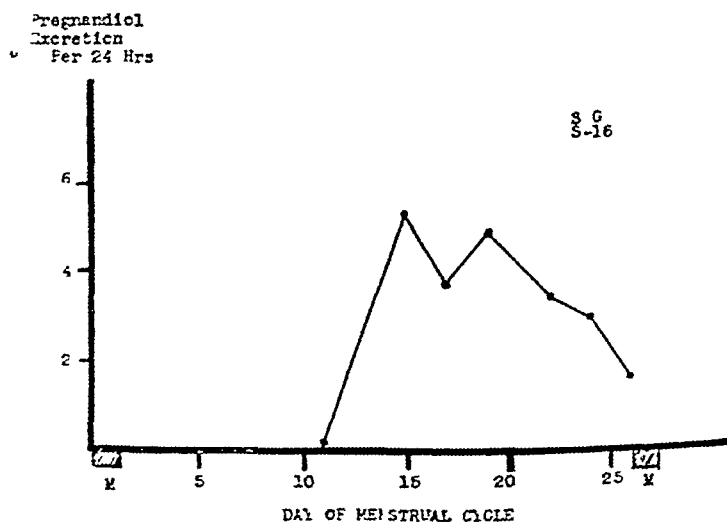


Fig. 4—Pregnan diol excretion in normal menstrual cycle (present method)

not consonant with those reported from this laboratory. Others have not only employed potassium dichromate as a standard but have read the colors at higher wave lengths (530 to  $570\text{ m}\mu$ ).<sup>11</sup> Since pregnandiol absorption in this region is minimal we feel that the authors were probably measuring substances other than pregnandiol.

The results of quantitative estimations in normal pregnancy in our laboratory are consistent with those obtained by others (Table II). The pattern and levels of pregnandiol excretion in the normal menstrual cycle coincide with

those reported by Venning and Browne<sup>14</sup> (see Fig. 4). These observations, which will be published elsewhere in extenso, further strengthen our conclusion that the method proposed is accurate, specific and practical for research and routine clinical use.

TABLE II. COMPARISON OF PREGNANDIOL EXCRETION VALUES IN NORMAL PREGNANCY

DAY(S)	PREGNANDIOL (MC IEP 24 HR.)	
98-106	5-11½	9-16½
106-114	6-20	15-38
114-122	10-25	19-36
122-130	13-31	27-46
130-138	22-32	21-62
138-146	44-72	30-72
146-154	48-81	41-91
154-162	55-95	56-108
162-170	60-101	50-101

The two sets of data given in this table are at first glance not strictly comparable. The Venning procedure calculates the pregnandiol concentration from NaPG which contains about 90 per cent of sodium pregnanolone glucuronide. The method here reported estimates pregnandiol only. It would therefore be expected that the pregnandiol values by the Venning method should be about 90 per cent higher than with the present method. The reason for the virtual coincidence of the values remains at present unexplained.

<sup>†</sup>Counted from the first day of the last normal menstrual period.

<sup>‡</sup>From Brown, Henry, and Venning; <sup>§</sup>Venning method (eight cases).

<sup>||</sup>This laboratory. Present method (four cases).

## SUMMARY

A method for the simple quantitative colorimetric determination of urinary pregnandiol is reported.

Spectrophotometric and melting point data are presented as evidence that pregnandiol alone is the substance measured.

Optimal conditions for the quantitative extraction of pregnandiol and color development are established.

Data presented indicate that an average of 95 per cent of the theoretic pregnandiol available is recovered by the procedure reported.

The results of pregnandiol determinations in the menstrual cycle and in normal pregnancy check closely with observations made in other laboratories which employed the longer gravimetric procedure.

We wish to thank Dr. Reimund Levine for his advice and encouragement in this work.

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## STUDIES IN SERUM PROTEINS

### II A RAPID CLINICAL METHOD FOR THE ACCURATE DETERMINATION OF ALBUMIN AND GLOBULIN IN SERUM OR PLASMA

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ELECTROPHORETIC analysis of serum has demonstrated that "serum albumin," as determined by the Howe sodium sulfate method, actually includes both albumin and alpha globulin.<sup>1,2</sup> Other methods for determining albumin values which approximate those obtained by electrophoresis have certain disadvantages. Precipitation of globulins by methanol as devised by Pillemer and Hutchinson,<sup>4</sup> is difficult technically since it involves working between 0 and 1° C and, even when these conditions are maintained, occasionally yields erratic results.<sup>1,2</sup> Popjak and McCarthy's method<sup>5</sup> for separating albumin and globulin with saturated magnesium sulfate involves a delay of twelve hours before filtration can be carried out. In addition, the albumin in the filtrate cannot be estimated by the biuret reaction but requires a Kjeldahl nitrogen determination. The 28 per cent sodium sulfate method of Milne<sup>6</sup> also requires an overnight delay for precipitation of the globulins. Chow has reported a quantitative immunochemical reaction which, however, employs biologic material of unstable titer and uncertain composition.

The method described below appears to overcome these difficulties. The procedure is based on our observation that sodium sulfite at a concentration of 26.88 per cent precipitates globulin from serum, plasma or plasma fractions. The earlier sodium sulfite method of Campbell and Hanna was adjusted to give results approximating Howe fractions.<sup>8</sup> Following precipitation of the globulin component immediate filtration yields a solution in which only albumin is found. The albumin in the filtrate may be determined by the biuret reaction of Weichselbaum<sup>9</sup> or by any other convenient method.

Analyses were performed on human plasma and on subfractions of plasma by the electrophoretic and sodium sulfite methods. The results obtained are compared in Tables I and II.

It will be seen from the results in Table I, obtained on plasma fractions that albumin is not precipitated by 26.88 per cent sodium sulfite to any extent whether in pure solution or combined with mixtures of globulins. All globulins were precipitated regardless of the distribution of globulin subfractions. Although fractions IV 4 and IV 6 contained large amounts of alpha globulin

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TABLE I COMPARISON OF ELECTROPHORETIC AND SODIUM SULFITE ANALYSIS OF PURIFIED PLASMA FRACTIONS\*

FRACTION	ANALYTIC METHOD	ALBUMIN† (GM %)	TOTAL GLOBULIN (GM %)	ALPHA GLOBULIN (GM %)	BETA GLOBULIN (GM %)	GAMMA GLOBULIN (GM %)
Albumin‡	Electrophoresis	4.90	0.00	0.00	0.00	0.00
	Sodium sulfite	4.90	0.00			
Gamma globulin§	Electrophoresis	0.00	5.30	0.00	0.00	5.30
	Sodium sulfite	0.00	5.30			
Mixture of albumin and gamma globulin	Electrophoresis	2.50	2.50	0.00	0.00	2.50
	Sodium sulfite	2.50	2.50			
IV 4	Electrophoresis	0.93	4.90	2.70	2.20	0.00
	Sodium sulfite	0.90	4.9			
IV 6	Electrophoresis	0.13	3.7	3.04	0.33	0.00
	Sodium sulfite	0.20	3.50			
IV 7	Electrophoresis	0.45	4.05	0.76	.69	0.00
	Sodium sulfite	0.75	3.75			
IV 8	Electrophoresis	0.87	0.43	0.30	0.13	0.00
	Sodium sulfite	4.05	0.25			
Average	Electrophoresis	1.85	2.94	0.91	0.91	1.11
	Sodium sulfite	1.90	2.89			

\*The electrophoretic analyses of purified plasma fractions quoted here are average results obtained for these fractions. The lyophilized fractions supplied were dissolved in 0.15M NaCl before analysis and filtered to remove residual turbidity. A sample of fraction VI could not be analyzed because the sodium sulfite filtrate was yellow and interfered with the accuracy of the photoelectric determination.

†Includes fast component.

‡Salt-free human albumin (Cutter Laboratories, Berkeley, Calif.) diluted with 0.15M NaCl.

§Immune human globulin (E. R. Squibb & Sons, New York, N. Y.) diluted with 0.15M NaCl.

||Sample contained 50 per cent salt-free human albumin and 50 per cent immune human globulin made up to a total protein concentration of 5.0 Gm. per 100 cc. with 0.15M NaCl.

TABLE II COMPARISON OF ELECTROPHORETIC AND SODIUM SULFITE ANALYSIS OF HUMAN SERA

DIAGNOSIS	ALBUMIN (CM %)			GLOBULIN (CM %)			A/C RATIO		
	FLECTIO PHOIF SIS	SODIUM SULFITE	DIFFERENCE BETWEEN FLECTIO PHOIF SIS AND SODIUM SULFITE	FLECTIO PHOIF SIS	SODIUM SULFITE	DIFFERENCE BETWEEN FLECTIO PHOIF SIS AND SODIUM SULFITE	FLECTIO PHOIF SIS	SODIUM SULFITE	DIFFERENCE BETWEEN FLECTIO PHOIF SIS AND SODIUM SULFITE
Multiple myeloma	2.02	2.00	+0.02	4.84	4.70	+0.14	0.42	0.43	-0.01
Multiple myeloma	1.97	2.30	-0.33	3.06	2.90	+0.16	0.64	0.79	-0.15
Multiple myeloma	1.80	1.50	+0.30	8.25	8.90	-0.65	0.22	0.17	+0.05
Rheumatic fever	1.65	1.75	-0.10	5.05	5.15	-0.10	0.33	0.34	-0.01
Rheumatic fever	2.10	3.10	0.00	5.14	5.40	-0.26	0.60	0.57	+0.03
Rheumatoid arthritis	2.10	2.20	-0.10	5.52	5.60	-0.08	0.38	0.39	-0.01
Hepatic cirrhosis	2.71	2.70	+0.01	5.85	6.00	-0.15	0.46	0.45	+0.01
Pooled normal serum	4.00	3.70	+0.30	3.40	3.25	+0.15	1.18	1.14	+0.04
Average	2.42	2.41		5.14	5.24		0.47	0.46	

which by the Howe method is included in the "albumin" fraction, sodium sulfite fractionations give good agreement with electrophoretic values

The human sera analyzed (Table II) consisted of six samples of known electrophoretic composition made available to us<sup>\*</sup> and two of our samples which were analyzed electrophoretically elsewhere†. Mean values for albumin globulin and A/G ratio did not differ from those obtained by electrophoresis by more than 2.2 per cent

Only one sample of pooled normal serum (Table II) was analyzed both electrophoretically and chemically. However a number of analyses have been carried out on pools of serum obtained from fifty to one hundred fifty donors. These analyses give an average value of 53 per cent albumin with individual pools varying from 51 to 55 per cent albumin. These values coincide well with the value of 52 per cent albumin obtained for plasma from normal adults by electrophoresis<sup>10</sup>

#### INTRODUCTION

##### Reagents —

Twenty eight per cent sodium sulfite solution. Dissolve exactly 28.00 Gm of anhydrous sodium sulfite in distilled water at 28° C. The salt is difficultly soluble but will go into solution with sufficient shaking. Make up to 100 ml and store at room temperature

Biuuret reagent (Weichselbaum)<sup>9</sup>

##### Determination —

- 1 Place exactly 24 ml of sodium sulfite solution in a 25 ml graduated mixing cylinder. Add 1.0 ml of serum or plasma and mix well by inversion. Do not shake.
- 2 Filter immediately through a double thickness of folded No. 42 Whatman filter paper. The filtrate should be crystal clear. If the first few drops are turbid they should be refiltered.
- 3 Discard the first 5 ml of filtrate. (A certain proportion of the albumin in the first few milliliters of filtrate will be adsorbed on the filter paper and low albumin values will be obtained if this material is used for analysis.)
- 4 To 5 ml of Weichselbaum's biuuret reagent in a cuvette add 5 ml of filtrate. Mix well by shaking.
- (The blank is prepared with 5 ml of biuuret reagent and 5 ml of 25 per cent sodium sulfite.)
- 5 After standing thirty minutes the solution is read on a photoelectric colorimeter or spectrophotometer at a wave length of 540 mμ.
- 6 The value obtained in the preceding step is that for serum albumin. Globulin is determined as the difference between total protein and albumin values.

<sup>\*</sup>Through the kindness of Dr. Philip P. Cohen of the Department of Physiological Chemistry, University of Wisconsin, Madison, Wis.

<sup>†</sup>With the cooperation of Mr. Miriam Reiner of the Bloch Medical Laboratory, Mount Sinai Hospital, New York, N. Y.

## SUMMARY

A method for the determination of albumin in serum, plasma, or plasma fractions is described. This method gives reproducible results which agree closely with those obtained by electrophoretic analysis. As the procedure is rapid and technically simple, it is suitable for use in the routine clinical laboratory.

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# SYSTEMATIC QUALITATIVE ANALYSIS OF BIOLOGIC MATERIALS FOR COMMON STEAM VOLATILE ORGANIC POISONS

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**T**OXICOLOGIC analysis of biologic material is sometimes required when no indication or circumstantial evidence of the presence or absence of a specific poison is available. While adequate systematic qualitative analytic procedures for inorganic poisons such as heavy metals are available, the search for organic poisons necessitates a laborious, time consuming, step by step analysis for each individual compound.

Organic poisons may be divided into the following groups: (1) steam volatile compounds, and (2) nonvolatile compounds extractable with suitable organic solvents from (a) aqueous acid solution and (b) aqueous alkaline solution.

An attempt has been made to develop methods for the systematic detection of various compounds found in each of these groups. In the present paper a simple scheme for the detection of the more common steam volatile poisons is described.

The following compounds are included in the scheme of analysis:

Acid steam distillate: phenol, cresol (tr cresol), thymol, aniline, hydrocyanic acid, chloroform, chloral hydrate, carbon tetrachloride, acetaldehyde, ethyl alcohol, formaldehyde, methyl alcohol, nitrobenzene, carbon disulfide.

Alkaline steam distillate: nicotine, amphetamine, aniline.

## EXPERIMENTAL

The acid steam distillate is obtained in the following manner. The tissue to be examined (100 Gm.) is homogenized in a Waring blender with distilled water (200 ml.) and the mixture is adjusted with tartaric acid to a pH of 5.0. The homogenate is transferred to a distilling flask (1 liter) which is part of an all glass steam distilling apparatus\*. About 5 Hengar boiling granules are added to the steam generating flask. All joints are lubricated with a thin layer of silicone. The distillate is collected in a suitable cooled container with the end of the condenser immersed in distilled water (10 ml.). The steam distillation is carried out until 200 ml. have been collected.

The homogenate is then made alkaline with magnesium oxide to a pH of 8.0. The steam distillation is repeated and 200 ml. of distillate again are collected.

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The apparatus designed for this procedure according to our specifications was obtained from E. Machlett & Sons, New York, N. Y.



Quantities of tissue weighing less than 100 Gm may be analyzed by employing proportionately smaller volumes of water in preparing the homogenate and by collecting appropriately reduced volumes of distillate. However, a minimum of 25 Gm of tissue is recommended for a complete analysis.

In the literature of this subject are described a large number of tests for the detection of the poisons considered in this scheme. However, many of these tests proved to be unsatisfactory, either because of insufficient sensitivity or because they gave positive or equivocal results with steam distillates from tissues containing no poison. Since each of those tests which was found suitable has been modified, a detailed description of each procedure is given. Reference is made to the papers in which the tests were originally described. The lowest concentration of the compound at which the test is definitely positive is listed. This concentration is considered the practical limit of sensitivity. None of the tests described give positive results with steam distillates from homogenates of liver containing no poison.

1 *Precipitation Test With Bromine Water*<sup>1</sup>—To the distillate (1 ml) add bromine water (1 drop). If no precipitation occurs, add additional reagent (2 or more drops). A positive reaction is indicated by the formation of a white or yellowish precipitate or by a definite cloudiness of the solution.

*Sensitivity* Phenol, 0.0025 per cent, trinitresol, 0.0020 per cent, thymol, 0.0015 per cent, aniline, 0.0015 per cent.

2 *Millon's Test*<sup>2</sup>—To the unknown solution (3 ml) in a small test tube add the reagent (3 drops). Heat the mixture at first gently, then with a gradually increasing intensity while observing carefully for changes in color. A positive reaction is indicated by a red or red-brown color or a precipitate. A bright yellow precipitate may form, this may consist of mercury salts and does not indicate a positive test.

*Sensitivity* Phenol, 0.0007 per cent, trinitresol, 0.0007 per cent, aniline, 0.0050 per cent.

3 *Prussian Blue Test for Cyanide*<sup>3, 4</sup>—To the unknown (3 ml) in a test tube add a solution of potassium hydroxide (5 per cent, 3 or 4 drops), then a freshly prepared solution of ferrous sulfate (2 per cent, 1 or 2 drops), and a solution of ferric chloride (1 per cent, 1 drop). Shake the solution and warm gently. Acidify carefully with hydrochloric acid (2N). If much hydrocyanic acid is present a precipitate of prussian blue (ferric ferrocyanide) will appear at once, but if the concentration is weak the colloidal solution will have merely a blue, blue green, or green-blue color. After some time a flocculent precipitate of prussian blue will settle to the bottom of the tube. Prussian blue will not appear if the reaction is alkaline. On the other hand, in strongly acid solutions the formation of prussian blue is delayed. Therefore, the final mixture should be adjusted (with indicator paper) to the optimal range of pH 2 to pH 5.

The limiting concentration of hydrocyanic acid in this test has been stated to be 1:50,000 (0.002 per cent)<sup>3</sup>, for practical purposes, however, the sensitivity is limited to a concentration of 0.004 per cent.

4 *Alkaline Pyridine Test* —To the unknown solution (2 ml) in a medium sized test tube add pyridine (0.4 ml reagent grade) and mix thoroughly. Add a solution of potassium hydroxide (40 per cent 4 ml) and heat one to three minutes in a boiling water bath while observing the pyridine layer for color changes. A red color developing in the pyridine layer within three minutes signifies a positive test. The color may fade with continued heating and it is essential therefore, to observe the reaction carefully.

*Sensitivity* Chloroform, 0.0002 per cent chloral hydrate 0.0001 per cent carbon tetrachloride, 0.025 per cent

5 *Direct p Hydroxydiphenyl Test* <sup>18b</sup>—To the distillate (1 drop) add a solution of copper sulfate (1 per cent 1 drop) and then concentrated sulfuric acid (1 ml). Immediately cool the tube with ice water and add a drop of the p hydroxydiphenyl reagent (p hydroxydiphenyl 1.5 per cent in a solution of sodium hydroxide 0.5 per cent). Shake the mixture thoroughly and allow it to stand for twenty minutes.

A blue color is formed in the presence of acetaldehyde; this changes gradually to bright violet (maximum color intensity occurs after twenty to thirty minutes). With formaldehyde a bright green color is produced but this changes to blue on standing.

*Sensitivity* Formaldehyde 0.0002 per cent acetaldehyde 0.0008 per cent

6 *p Hydroxydiphenyl Test Following Oxidation* <sup>6</sup>—In a large test tube place the distillate (0.1 ml) a solution of potassium permanganate (5 per cent 1 drop) and phosphoric acid (10 per cent 1 drop). After mixing the solution is allowed to stand at room temperature for one minute. Add a solution of oxalic acid (saturated) drop by drop, with shaking until the solution is decolorized. After decolorization has occurred add concentrated sulfuric acid (6 drops) while agitating the tube in ice water. Remove the tube from the bath add a solution of copper sulfate (1 per cent 1 drop) and mix. Replace the tube in ice water and agitate it while adding concentrated sulfuric acid (3 ml). Remove the tube from the bath, add a drop of the p hydroxydiphenyl solution (as in Test 5), mix well and allow to stand at room temperature. Record any production of color after twenty to twenty five minutes.

In the presence of methyl alcohol a bright green blue to blue color is produced while with ethyl alcohol a bright blue violet color appears.

*Sensitivity* Methanol, 0.0008 per cent ethanol 0.004 per cent

7 *Indophenol Test* —To the unknown solution (3 ml) slowly add concentrated  $H_2SO_4$  (3 ml) while cooling the tube in a beaker of water. Then add a small amount of sodium nitrite and mix. Allow to stand at room temperature for five minutes and observe the color.

In the presence of phenol in a concentration of 0.01 per cent or higher a red color is produced. In a still higher concentration (1/1000) other phenols also produce colors: cresol yellow brown thymol faint yellow.

8 *Ware's Test for Phenol*—To a portion of the distillate (1 ml) add concentrated HCl (3 ml). Mix and warm in a water bath at 70° C for one minute. Quickly add a small amount of Ware's mixture (sodium nitrite, 1 part, sodium nitrate, 1 part, anhydrous sodium sulfate, 2 parts) and let stand at room temperature for five minutes. Observe the color. Cool, make alkaline with a concentrated solution of  $\text{NH}_4\text{OH}$ , and again observe the color.

In the presence of phenol a cherry red color is produced in acid solution and a green color in alkaline solution. The test is sensitive to at least 1 ml of 0.02 per cent phenol. Thierisol and aniline produce yellow or brown colors, but these are readily distinguished from the colors observed in the presence of phenol.

9 *Selenous Acid Test*<sup>9</sup>—To a portion of freshly prepared Mecke's reagent (25 ml) add the distillate (0.5 ml). Mix and observe the color at once (Mecke's reagent is prepared by dissolving 0.5 Gm of selenous acid in 100 ml of concentrated sulfuric acid).

In the presence of thierisol a distinct red color is produced, whereas phenol is at first yellow green and gradually becomes red-brown.

*Sensitivity* Thierisol 0.005 per cent.

10 *Hypochlorite test*<sup>10</sup>—To the distillate (5 drops), in a depression of a white spot plate add chemically pure aqueous sodium hypochlorite solution (2 drops) and observe any change in color.

If aniline is present in concentrations greater than 0.0025 per cent, a clear violet, gradually changing to a dirty violet color, is produced.

11 *Diazotization and Coupling*<sup>11</sup>—The distillate (1 drop), in a depression of a white spot plate is acidified with dilute HCl (1 small drop). Add a freshly prepared solution of sodium nitrite (0.2 per cent, 1 drop) and mix. Destroy the excess nitrous acid with a solution of ammonium sulfamate (1 per cent, 1 drop). Then add freshly prepared coupling reagent, N-(1-naphthyl) ethylene diamine dihydrochloride (0.2 per cent, 1 drop), and observe the development of color during a period of one to two minutes.

Aniline (and all other primary amines) form diazo compounds with nitrous acid, these are converted to highly colored azo compounds with N-(1-naphthyl)-ethylenediamine. With aniline a purple color is produced which becomes gradually more intense within about one minute after addition of the coupling agent. Phenol, cresol, amphetamine, and nicotine are negative. The test is sensitive to 0.00065 per cent aniline.

12 *Test With Wasicky's reagent*<sup>12</sup>—To the distillate (1 ml) add quickly, with mixing, an equal volume of the reagent ( $\beta$ -dimethylaminobenzaldehyde, 2 Gm, in concentrated  $\text{H}_2\text{SO}_4$ , 6 ml, to which water, 0.4 ml, is added). Observe the color immediately.

In the presence of thymol an orange to orange-red color is produced whereas the control (distilled water) tested in the same manner has a pale yellow color.

The test is positive to 0.001 per cent thymol. The orange color fades quickly.

13 *Fulton's Test for Phenols*<sup>13</sup>—To the distillate (5 ml) add  $\text{NH}_4\text{OH}$  (28 per cent, 5 drops) hydrogen peroxide (3 per cent 1 ml), and a solution of copper sulfate (0.1 per cent, 3 drops). Mix and let stand for ten minutes. In the presence of thymol a pink violet color is produced.

This test is not very sensitive. The absolute limit of sensitivity is a thymol concentration of 0.005 per cent. Other phenols also produce colors; for example, a solution of tricresol produces an orange pink color which changes to yellow.

14 *Thiocyanate Test*<sup>14</sup>—To the distillate (3 ml) in a test tube add  $\text{KOH}$  (2N, 1 drop) and a solution of yellow ammonium sulfide (a solution of 10 per cent ammonium hydrosulfide saturated with hydrogen sulfide 2 drops). Heat gently for two minutes over the flame and add  $\text{HCl}$  (4N, 0.5 ml). Allow the solution to cool, add a solution of ferric chloride (1 per cent, 3 to 5 drops). A red or red brown color indicates the presence of cyanide.

The test is sensitive to 3 ml of a 1:70,000 solution of cyanide (calculated as  $\text{HCN}$ ).

15 *Picric Acid Test*<sup>1</sup>—To the distillate (3 ml) add a solution of picric acid (saturated, 3 drops) and a solution of sodium carbonate (20 per cent, 3 drops). Warm gently over the flame for a few minutes, allow to stand at room temperature for ten minutes. An orange red color or precipitate indicates a positive test.

The test is sensitive to 3 ml of a 1:250,000 cyanide solution (calculated as  $\text{HCN}$ ).

16 *Resorcinol Test (Strong Alkali)*<sup>16</sup>—To the distillate (1 ml) add aqueous resorcinol solution (saturated 2 drops) and a solution of  $\text{KOH}$  (20 per cent, 1 ml). Warm at  $60^\circ\text{C}$  in a water bath for two to three minutes and observe the color of the solution. A red color is produced with chloroform, chloral hydrate, and carbon tetrachloride.

*Sensitivity* Chloroform, 0.005 per cent; chloral hydrate, 0.002 per cent; carbon tetrachloride, 0.025 per cent.

17 *Resorcinol Test (Weak Alkali)*<sup>16</sup>—To the distillate (1 ml) add resorcinol solution (saturated 2 drops) and a solution of sodium carbonate (20 per cent, 1 ml). Shake and let stand for twenty minutes at room temperature. Dilute with distilled water (2 or 3 volumes) and observe the tube for green fluorescence. Viewing conditions must be optimal or the fluorescence may be missed. View against a black background with light directed toward the tube at an angle of  $90^\circ$  degrees from the line of vision.

A solution of chloral hydrate in a concentration of 0.001 per cent is sufficient to give a positive test, whereas chloroform and carbon tetrachloride are negative.

18 *Phloroglucinol Test*<sup>1</sup>—To the distillate (1 ml) add a small amount of granular phloroglucinol (chemically pure) and a solution of sodium carbonate (20 per cent, 1 ml). Shake and observe the color during a period of one half hour at room temperature.

A solution containing as little as 0.001 per cent chloral hydrate will produce an orange-red color. The color of the control is pale-violet. Chloroform and carbon tetrachloride do not give this test.

19 *Chromotropic Acid Test*<sup>18a</sup> (*According to Fiegl*<sup>18b</sup>)—The distillate (0.05 ml or 1 drop), in a test tube, is mixed with a solution of sulfuric acid (72 per cent, 3 ml). A little solid chromotropic acid (1,8-dihydroxy naphthalene 3,6-disulfonic acid) is added and the tube is heated in a water bath at 60° C for ten minutes. A bright violet color appears in the presence of formaldehyde. The test will detect formaldehyde in a 0.0007 per cent solution. Acetaldehyde is negative.

20 *Fuchsin Test*<sup>18b, 19</sup>—To the distillate (1 ml) add reduced fuchsin reagent (10 drops) and concentrated  $H_2SO_4$  (5 drops). Let stand at room temperature for twenty minutes. The presence of formaldehyde is indicated by a violet color.

*Sensitivity* Formaldehyde, 0.001 per cent, acetaldehyde is negative.

The reagent is prepared as follows. Dissolve basic fuchsin (2 Gm) in hot water (120 ml) and allow the solution to cool. Add a solution of sodium sulfate (2 Gm) in distilled water (20 ml) and follow with concentrated HCl (2 ml). Dilute with distilled water to 200 ml and let stand at least one hour. The freshly prepared solution has a yellow color and should be stored in a cold room.

21 *Oxidation Tests for Methanol*<sup>18b, 19</sup>—To the distillate (5 ml) add a solution of potassium permanganate (5 per cent, 3 drops) and a solution of phosphoric acid (10 per cent, 4 drops). Let stand at room temperature for two minutes. Add small amounts of sodium bisulfite, with mixing, until the solution is decolorized (avoid excessive quantities).

*Divide the solution in two parts*

(a) To the oxidized solution (3 ml) add reduced fuchsin reagent (15 drops) and concentrated  $H_2SO_4$  (6 drops). Mix and let stand for twenty minutes. If methanol is present in the original solution in a concentration of 0.008 per cent or higher a violet color will appear.

(b) To the oxidized solution (1 ml) add  $H_2SO_4$  (72 per cent, 4 ml) and a small amount of chromotropic acid. Warm at 60° C for ten minutes (see Test 19).

*Sensitivity* Methanol, 0.0013 per cent.

22 *Electrolytic Reduction of Nitrobenzene and Detection of the Nitroso Compound Produced*<sup>18b, 20</sup>—To the distillate (1 ml) in a small (10 ml) beaker add the freshly prepared reagent (1 per cent solution of sodium pentacyanoamine ferriate, 1 ml)<sup>-1</sup> and a solution of sodium hydroxide (4N, 1 ml). A current is then allowed to pass through the solution using a nickel wire as a cathode and a lead wire as an anode. The source of the current can be either a flashlight battery or a 4-volt storage battery. Allow the electrolysis to proceed for one-half hour. If nitro compounds are present in the original solution

a color will appear during the electrolysis nitrobenzene producing a bright green color varying in intensity with the concentration. With high concentrations a dark violet color may be produced.

*Sensitivity* Nitrobenzene, 0.006 per cent

23 *Reduction-Diazotization Test*<sup>11</sup>—To the distillate (6 drops) in a test tube add HCl (2N, 3 drops) and a small amount of zinc powder. Agitate and allow to stand for two to three minutes at room temperature. Remove two drops with a dropper and place them in a depression of a white spot plate, then perform the diazotization test (Test 11).

In this test nitrobenzene is reduced to aniline which is detected by diazotization and coupling. The test is sensitive to a solution of 0.006 per cent nitrobenzene.

24 *Formaldehyde Plumbite Test*<sup>15b, 2</sup>—To the distillate (2 ml) add bromine water, drop by drop, until the light yellow color is permanent. Let stand for two minutes. In this way hydrogen sulfide which interferes with the test, is removed by oxidation. Remove the excess bromine by adding a small amount of sodium sulfite. Then add formaldehyde (40 per cent, 5 drops) and alkaline plumbite solution (5 drops). (Prepare the solution by dissolving lead acetate 1 Gm, in water 100 ml, and adding KOH 20 per cent until the precipitate which forms has dissolved.) Let stand for one half hour. If large amounts of carbon disulfide are present a black or dark brown precipitate will form while with smaller amounts the solution is colored yellow brown.

*Sensitivity* Carbon disulfide, 0.004 per cent

25 *Ammonium Molybdate Test*<sup>2, 16</sup>—To the distillate (2 ml) add alcoholic potassium hydroxide (10 per cent, 0.5 ml) and heat in a boiling water bath for five minutes. Cool to room temperature then add ammonium molybdate solution (2 drops) and acidify with concentrated sulfuric acid added drop by drop. In the presence of carbon disulfide a blue or green blue color develops which, with very small amounts of carbon disulfide, may form only very slowly. Therefore, allow the tube to stand for two hours at room temperature. A greenish yellow color may develop in the distilled water control.

*Sensitivity* Carbon disulfide, 0.004 per cent

26 *Precipitation With Nessler's Reagent*<sup>3</sup>—To the distillate from the alkaline solution (5 ml) add Nessler's reagent (10 drops). If a precipitate does not appear at once, prick the tube in ice or keep in the refrigerator for at least four hours and again examine for a precipitate.

In a concentration of 0.1 per cent, amphetamine produces an amorphous white precipitate. In a concentration of 0.02 per cent a white cloud appears. Aniline and nicotine are not precipitated even at concentrations higher than 0.1 per cent.

27 *p-Nitroaniline Test*<sup>4</sup>—The alkaline distillate (15 ml) is extracted with three portions (5 ml each) of a mixture of petroleum ether-toluene (1:1) by shaking for five minutes with each extraction. The amine is transferred to

aqueous solution by washing the combined ether-toluene fractions with two portions of HCl (0.2 per cent, 5 ml each). The acid solution is carefully adjusted to pH 6 to pH 7 with dilute NaOH solution. To the resulting solution (5 ml) add cold p-nitrobenzenediazonium chloride reagent (5 ml) and allow to stand for an hour at room temperature. Add a solution of sodium carbonate (11 per cent, 5 ml), let stand for fifteen minutes, and add sodium hydroxide (10 per cent, 1 ml) to develop the color.

In the presence of amphetamine a distinct red color is produced. Aniline may also produce a red color even when very small amounts are present. Nicotine, however, is negative to this test or may produce a nondescript greenish yellow color. A solution of 0.001 per cent amphetamine (sulfate) may be detected.

**Stock-Solution** p-Nitroaniline (3.5 Gm) is suspended in concentrated HCl (5 ml) by breaking up clumps with a glass rod. The suspension is diluted with distilled water to 500 ml, shaken for twenty minutes, and filtered. The filtrate remains stable when kept in the refrigerator.

TABLE I. GROUP REACTIONS FOR POISONS IN THE ACID STEAM DISTILLATE

Group I—Positive to bromine water test (1)		Group II—Negative to bromine water test (1)	
Aniline	Cresol (Tricresol)	Hydrocyanic acid	Formaldehyde
Phenol	Thymol	Chloroform	Nitrobenzene
Millon's test (2)		Carbon tetrachloride	Methyl alcohol
		Chloral hydrate	Carbon disulfide
		Acetaldehyde	
		Ethyl alcohol	
Positive		Prussian blue test (3)	
Group I A			
Aniline		Positive—Group II A	
Phenol		Hydrocyanic acid	
Cresol		Negative—Group II B	
		All others	
		Direct alkaline pyridine test (4)	
Positive—Group II B 1		Negative—Group II B 2	
Chloroform		Acetaldehyde	
Chloral hydrate		Ethyl alcohol	
Carbon tetrachloride		Formaldehyde	
(in concentration of 1:5,000 or higher)		Methyl alcohol	
		Nitrobenzene	
		Carbon disulfide	
		Direct p-hydroxydiphenyl test (5)	
Positive—Group II B 2a		Negative—Group II B 2b	
Acetaldehyde		p-Hydroxydiphenyl test following oxidation (6)	
Formaldehyde			
Positive—Group II B 2b 1		Negative—Group II B 2b 2	
Methyl alcohol		Nitrobenzene	
Ethyl alcohol		Carbon disulfide	

*Reagent* The stock solution (10 ml) is cooled ( $0^{\circ}\text{C}$ ), concentrated HCl (2 ml) is added and the mixture is allowed to stand at  $0^{\circ}\text{C}$  for ten minutes. Freshly prepared sodium nitrite solution (0.7 per cent, 6 ml) is added, and after another ten minutes at  $0^{\circ}\text{C}$  the solution is diluted with distilled water to 200 milliliters. The solution is ready for use after it has remained at  $0^{\circ}\text{C}$  for two hours. When stored in a cold room it remains stable for three weeks.

**28 Picric Acid Precipitation Test**<sup>2</sup>—To the distillate (5 ml) add picric acid solution (saturated, 10 drops) and mix. Allow to stand in ice or in the refrigerator for four to six hours. If a precipitate forms, examine it under the microscope.

If nicotine is present characteristic crystals of the picrate will appear. These are long thin, yellow needles which grow in fan shaped clusters resembling sheaves of grain.

*Sensitivity* Nicotine, 0.0025 per cent.

Aniline and amphetamine in much higher concentrations may be precipitated by picric acid but the precipitate is not characteristic in appearance.

**29 Oxidation Nitroprusside Test**<sup>26</sup>—To the distillate (5 ml) add 2 drops of a buffer solution of pH 4.0 (72 ml of molar acetic acid, 12 ml of molar sodium hydroxide), potassium persulfate (0.1 to 2 Gm) and a solution of silver nitrate (1 per cent, 1 drop). Place in a boiling water bath for one minute, shake well and cool at  $20^{\circ}\text{C}$ . Add crystalline sodium thiosulfate (0.05 to 0.15 Gm) shake until dissolved, add sodium nitroprusside (10 per cent 2 to 4 drops), and make slightly alkaline with ammonium hydroxide solution (3 per cent).

In the presence of nicotine a red color appears in two to three minutes. The solution must be slightly acid and must be free from ions which precipitate silver ions. The presence of aniline produces a violet discoloration during the heating but the color soon changes to yellow. With larger amounts of aniline the solution becomes black and interferes with the test. Amphetamine does not produce a color.

*Sensitivity* Nicotine, 0.0025 per cent.

#### DETECTION OF POISONS IN THE ACID AND ALKALINE STEAM DISTILLATES

In Table I the poisons in the acid steam distillate are classified according to group reactions. (The figures in parentheses refer to the number under which the test is described in the preceding section of this paper.) After a poison has been classified by the group reaction its presence is ascertained by further differentiating and confirmatory tests (Table II).

#### DISCUSSION

Phenolic compounds such as salicylic acid,  $\alpha$  and  $\beta$  naphthol and pyrocatalol are not included in this scheme since in disagreement with certain text books of toxicology<sup>2</sup> it was found that these compounds are not steam volatile to any significant degree even if the pH of the solution to be distilled is as low as 2.0.



TABLE II DIFFERENTIATING TESTS—ACID STEAM DISTILLATE

Group I A						
	INDOPHENOL TEST (7)	WARE'S TEST (8)		SELENOUS ACID TEST (9)	HYPOCHLORITE TEST (10)	DIAZOTIZATION AND COUPLING (11)
		ACID SOLUTION	ALKALINE SOLUTION			
Phenol	Red, Red brown	Cherry red	Green	Pale yellow	Neg	Neg
Tricresol	Light brown	Dark amber	Yellow	Red amber	Neg	Neg
Aniline	Pale yellow or colorless	Yellow	Bright yellow	Pale yellow	Violet	Bright purple
Group I B						
Thymol Test with Waseky's reagent (12)—orange red color						
Fulton's test for phenols (13)—pink violet color						
Group II A						
Hydrocyanic acid						
Thiocyanate test (14)—red or red brown color						
Picric acid test (15)—orange red color or precipitate						
Group II B 1						
	RESORCINOL TEST (16) STRONG ALKALI		RESORCINOL TEST (17) WEAK ALKALI		PHLOROGLUCINOL TEST (18)	
	Bright red		Green fluorescence		Lilac, changing to orange, to red	
Chloral hydrate						
Chloroform	Red, orange red		No fluorescence		Neg	
Carbon tetrachloride	Red, orange red		No fluorescence		Neg	
Group II B 2a						
	CHROMOTROPIC ACID TEST (19)				FUCHSIN TEST (20)	
	Neg Pos				Neg Pos	
Acetaldehyde						
Formaldehyde						
Group II B 2b 1						
	OXIDATION (21) FOLLOWED BY CHROMOTROPIC ACID TEST (19)				FUCHSIN TEST (20)	
	Pos Neg				Pos Neg	
Methyl alcohol						
Ethyl alcohol						
Group II B 2b 2						
	ELECTROLYTIC REDUCTION (22)		REDUCTION DIAZOTIZATION (23)		FORMALDEHYDE PLUMBITE TEST (24)	
	Green color Neg, yellow color of reagent		Pos Neg		Neg Yellow, brown, or black precipitate or solution	
Nitrobenzene						
Carbon disulfide					Neg Blue or blue green color	

TABLE III DIFFERENTIATING TESTS—ALKALINE STEAM DISTILLATE

	NESSLER'S TEST (26)	p-NITRO ANILINE TEST (27)	PICRIC ACID TEST (28)	OXIDATION NITROPRUS SIDE TEST (29)	DIAZOTIZATION AND COUPLING (11)	HYPOCHLORITE TEST (10)
Amphetamine	Pos	Pos	Amorphous or no precipitate	Neg	Neg	Neg
Nicotine	Neg	Neg	Characteristic needle shaped crystals	red color	Neg	Neg
Aniline	Neg	Pos	Amorphous or no precipitate	Neg or black	Pos	Pos

Tests for the presence of aniline should be carried out in both the acid and alkaline steam distillates since this compound is steam volatile at both pH 5.0 and pH 8.0

Carbon tetrachloride is positive to the tests listed for chloroform, but only in much higher concentrations. Therefore, if these tests are positive, the possibility of the presence of carbon tetrachloride should be kept in mind. However, if the steam distillate is positive to these tests and does not have the odor of carbon tetrachloride, it is likely to contain chloroform.

Acetone is not included in the scheme. Since ketone bodies are normal intermediaries of fat metabolism, steam distillates of homogenates from tissues (such as liver and kidney) regularly give positive reactions if sensitive tests for acetone are performed.

#### SUMMARY

An analytic scheme for the detection of the more common steam volatile organic poisons is described.

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# A METHOD FOR THE DETERMINATION OF TITERS BETWEEN 10 AND 100 IN THE QUANTITATIVE COMPLEMENT FIXATION TEST FOR SYPHILIS

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THE value of quantitative tests in diagnosis and control of treatment of syphilis is now widely appreciated. The complement fixation test developed in this laboratory is based on essential quantitative principles<sup>1,2</sup> and in practice yields an accurate and reproducible index of titer<sup>10,14</sup>. Studies have long been directed to a simplification of the method that would not sacrifice the advantages of an optimally adjusted system. The present report describes an abbreviated technique in which four or six dilutions of the specimen are titrated with six 50 per cent units of complement. The final values are read directly from tables. The method is well suited to the testing of sera from patients under treatment for primary or secondary syphilis and indeed for all but an occasional specimen.

The rationale and technique for determining titers greater than 10 in the quantitative complement fixation test for syphilis have been described in previous publications<sup>1,10</sup>. The procedure<sup>1</sup> first used employed a graphic expression of the results, on the basis of which titers were determined by linear extrapolation. This method required testing several dilutions of a specimen with varying amounts of antigen and 3, 6, 9 and 12 units of complement and was applicable to specimens of all degrees of reactivity. Titers from slightly over 10 that is 11 or 12 to titers of 300 to 500 and even up to 2 000 were obtained. A procedure in which 6 and 9 units of complement were used each with a single amount of antigen was later found to simplify the technique considerably without great sacrifice of accuracy<sup>16,17</sup>. The graphic method of estimating titers was supplanted by tables of calculated values. Experience with this method in testing sera from patients under treatment for primary and secondary syphilis indicated that titers up to 100 furnish as much information as is required in the management of such cases. The procedure here described was designed primarily to determine titers in the range of 10 to 100. It can nevertheless be extended readily to determine higher titers.

A preliminary titer range test is performed to select the amounts of serum to be tested.

*Technique of the Titer Range Test for Use With Serum*—The technique of the titer range test differs slightly from the one previously described<sup>18</sup>. Proceed as indicated in Table I. Prepare the 1:5 dilution of the reacting serum to be tested using as a diluent pooled human sera that are clear and colorless, are not anticomplementary and have failed to react in serologic tests for syphilis. Use

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this diluent also to adjust the volume of serum to 0.05 ml in tube 2. Inactivate the diluent for thirty minutes each day before use. Controls of the anticomplementary activity of the specimen are included in Table I but are not essential. They are of value in estimating the approximate titer.

TABLE I PRELIMINARY TITER RANGE TEST OF SERUM

TUBE	REACTING SERUM		EQUIVALENT AMOUNT OF SERUM (ML)	POOLED NON REACTING SERA (ML)	CARDIO LIPIN ANTIGEN #72 DILUTED 1:67 (ML)	UNITS OF COMPLEMENT PER 0.1 ML (ML)		SALT SOLUTION (ML)	PERIOD OF FIXATION	SENSITIZED SHEEP CELLS (ML)	PERCENT HEMOLYSIS
	DILUTION	AMOUNT PIPETTED (ML)				1	6				
1	1:5	0.05	0.01		0.1		0.1	0.05	4 hr, 36°C	0.2	100%
2	1:5	0.02	0.004	0.03	0.1		0.1	0.05		0.2	
3	Undiluted	0.05	0.05			0.1		0.15		0.2	
4	Undiluted	0.05	0.05			0.2		0.05		0.2	

Tubes 3 and 4 can be omitted if an estimated titer is not desired.

The titer is expressed as the ratio of the amount of complement required to give 50 per cent hemolysis with 0.05 ml of serum (or 0.2 ml of cerebrospinal fluid) and an optimal dose of antigen to the amount required to give 50 per cent hemolysis with the same amounts of the specimen alone. For convenience, the findings with specimen and antigen are referred to as (S+A) and with the specimen alone, as (S). The titer is the ratio  $\frac{(S+A)}{(S)}$ , and is expressed in two significant figures. Record the percentage of hemolysis in each tube by comparison with a color standard.<sup>161</sup> Determine the values of (S+A) by reference to Table II.

TABLE II VALUES OF (S+A)\* FOR TESTS WITH 6 UNITS OF COMPLEMENT, ANTIGEN, AND DILUTED SERUM

PER CENT HEMOLYSIS	SERUM DILUTED TO 1									
	25	30	40	50	60	75	95	120	150	200
	EQUIVALENT ML OF UNDILUTED SERUM									
5	0.02	0.0167	0.0125	0.01	0.0083	0.0067	0.0053	0.004	0.0033	0.0025
10	23	27	36	46	55	68	86	114	137	160
15	21	25	33	42	50	62	79	104	125	170
20	20	23	31	39	47	59	74	98	117	160
25	19	22	30	37	44	56	70	93	111	150
30	18	22	29	36	43	54	68	90	108	140
35	17	20	27	34	41	51	65	85	102	130
40	17	20	27	34	40	50	64	84	101	130
45	16	19	26	32	38	48	61	80	96	120
50	16	19	25	31	37	47	59	78	93	120
55	15	18	24	30	36	45	57	75	90	120
60	15	17	23	29	35	44	55	73	87	110
65	14	17	22	28	34	42	53	70	84	110
70	14	16	22	27	32	41	51	68	81	100
75	13	16	21	26	31	39	49	65	78	95
80	12	15	20	25	29	37	47	61	74	94
85	12	14	19	24	28	35	45	59	71	94
90	11	13	18	22	26	33	42	55	66	85
95	10	12	16	20	24	30	38	50	60	80

\* (S+A). The units of complement required for 50 per cent hemolysis with specimen and antigen (Table IX, line 4) multiplied by the dilution factor.

*Interpretation of the Titer Range Test With Serum*—When complete hemolysis is obtained with both amounts of serum tested, the value for (S+A) is less than 20, and amounts from 0.02 through 0.01 ml should be tested to determine the exact titer (see Table II). Five hundredths of a milliliter of serum diluted from 1:2.5 through 1:5 are used in the test.

When partial hemolysis is obtained with either amount in the titer range test, determine from Table II the amounts of serum and the sequence of dilutions to use, for example, if 10 per cent hemolysis occurs with 0.01 ml and complete hemolysis with 0.004 ml, a value between 42 and 50 is indicated. Therefore, test the serum diluted from 1:4 through 1:12.5. These dilutions include one amount above and one below the range of values indicated for (S+A) to allow for possible variations in complement.

When no hemolysis is obtained in either amount in the titer range test, the value of (S+A) is greater than 100. If the exact titer is desired in such instances titrate amounts smaller than 0.004 milliliter. For this purpose, use a series of dilutions ten times as great as those given in Table II.

Table III indicates the amounts of reacting and nonreacting serum used to prepare the dilutions.

TABLE III AMOUNTS OF REACTING AND NONREACTING SERUM USED IN THE PREPARATION OF DILUTIONS

DILUTION	REACTING SERUM (ML)	NONREACTING SERUM (ML)	REACTING SERUM CONTAINED IN 0.05 ML OF EACH DILUTION (ML)	REMARKS
1:2.5	0.08	0.12	0.02	Higher dilutions in
1:3	0.07	0.14	0.0167	the same progres
1:4	0.05	0.15	0.0125	sion can be pre
1:5	0.05	0.2	0.01	pared for determin
1:6	0.04	0.2	0.0083	ing titers greater
1:7.5	0.04	0.26	0.0067	than 100. Do not
1:9.5	0.02	0.17	0.0053	pipette less than
1:12.5	0.02	0.23	0.004	0.02 ml of reacting
1:15	0.02	0.28	0.0033	serum to prepare
1:20	0.02	0.38	0.0025	dilutions for these tests

*Technique of the Titer Range Test for Use With Cerebrospinal Fluid*—When the titer of the cerebrospinal fluid is greater than 10, perform a titer range test as outlined in Table IV. Use Table V for determining the value of (S+A). Depending upon the range of reaction indicated by this test, use in the final titration, varying amounts of a 1:2 dilution made with salt solution in the sequence of amounts given in Table V. If titers greater than 100 are desired use a 1:20 dilution.

*Technique for Determination of Titers Between 10 and 100 for Both Serum and Cerebrospinal Fluid*—The method of testing is illustrated in Tables VI and VII, and typical results are shown in Table VIII. The calculated values for (S+A) corresponding to the degrees of hemolysis obtained in the test of the different amounts of the specimen used are shown in Tables II and V. These

TABLE IV PRELIMINARY TITR RANGE TEST OF CEREBROSPINAL FLUID

TUBE	UNDILUTED CEREBROSPINAL FLUID (ML)	SALT SOLUTION (ML)	CARDIOLIPIN ANTIGEN #72 DILUTED 1:90 (ML)	UNITS OF COMPLEMENT PEP 0.1 ML (ML)		PERIOD OF FIXATION	SENSITIZED SHEEP CELLS (ML)	PERIOD OF HEMOLYSIS
				1	6			
1	0.04	0.16	0.1	0.1	0.1	4 hr, 36° C	0.2	15 min, 37° C
2	0.02	0.18	0.1	0.1	0.1		0.2	
3	0.2	0.1		0.2			0.2	
4	0.2						0.2	

Tubes 3 and 4 can be omitted if an estimated titer is not desired

TABLE V VALUES FOR (S+A)\* IN TESTS WITH ANTIGEN, 6 UNITS OF COMPLEMENT, AND CEREBROSPINAL FLUID IN THE GIVEN AMOUNTS

PER CENT HEMOLYSIS	AMOUNTS OF CEREBROSPINAL FLUID DILUTED TO 1:2									
	0.16	0.13	0.1	0.08	0.07	0.06	0.05	0.04	0.03	0.02
	EQUIVALENT AMOUNTS OF UNDILUTED CEREBROSPINAL FLUID									
	0.08	0.065	0.05	0.04	0.035	0.03	0.025	0.02	0.015	0.01
5	23	28	36	46	52	61	73	91	121	150
10	21	25	33	42	47	55	66	83	110	140
15	20	24	31	39	45	52	62	78	104	130
20	19	23	30	37	42	49	59	74	98	120
25	18	22	29	36	41	48	58	72	96	110
30	17	21	27	34	39	45	54	68	90	100
35	17	21	27	34	38	45	54	67	89	100
40	16	20	26	31	37	43	51	64	85	100
45	16	19	25	31	35	41	50	62	80	100
50	15	18	24	30	34	40	48	60	77	110
55	15	18	23	29	33	39	46	58	74	110
60	14	17	22	28	32	37	45	56	72	100
65	14	17	22	27	31	36	44	52	69	100
70	13	16	21	26	30	35	42	49	65	94
75	12	15	20	25	28	33	39	47	63	88
80	12	14	19	24	27	31	38	44	59	80
85	11	14	18	22	25	29	35	40	53	80
90	10	12	16	20	23	27	32			

\* (S+A) The units of complement required for 50 per cent hemolysis with specimen and antigen (Table IX line 4) multiplied by the dilution factor

TABLE VI DETERMINATION OF TITERS OF SERUM UP TO 100

DILUTION OF SPECIMEN	AMOUNT PIPETTED (ML)	CARDIOLIPIN ANTIGEN #72 DILUTED 1:67 (ML)	UNITS OF COMPLEMENT PEP 0.1 ML (ML)		SALT SOLUTION (ML)	PERIOD OF FIXATION	SENSITIZED CELLS (ML)	PERIOD OF HEMOLYSIS
			1	6				
Undiluted	0.05		0.1		0.15	4 hr, 36° C	0.2	15 min, 37° C
Undiluted	0.05		0.1		0.15		0.2	
Undiluted	0.05		0.2		0.05		0.2	
1st dilution	0.05	0.1		0.1	0.05		0.2	
2nd dilution	0.05	0.1		0.1	0.05		0.2	
3rd dilution	0.05	0.1		0.1	0.05		0.2	
4th dilution	0.05	0.1		0.1	0.05		0.2	
5th dilution	0.05	0.1		0.1	0.05		0.2	
6th dilution	0.05	0.1		0.1	0.05		0.2	

values were obtained by multiplying the units of complement required for 50 per cent hemolysis in the presence of the diluted specimen and the maximum reacting dose of antigen, as read from line 4 of Table IX, by the dilution factor,

that is, 0.05 ml divided by the milliliters of serum used. For example in a test in which 65 per cent hemolysis occurs with 0.05 ml of a 1:5 dilution of serum, (S+A) would be  $5.4 \times \frac{0.05}{0.01}$  or 27. Similarly in a test of 0.02 ml of cerebrospinal fluid 40 per cent hemolysis would correspond to an (S+A) value of  $6.4 \times \frac{0.2}{0.02}$ , or 64.

TABLE VII DETERMINATION OF TITERS OF CEREBROSPINAL FLUID UP TO 100

DILUTION OF SPECIMEN	AMOUNT PIPETTED* (ML.)	SALT SOLUTION† (ML.)	CARDIO LIPIN ANTIGEN #72 DILUTED 1:90 (ML.)	UNITS OF COMPLEMENT PER 0.1 ML (ML.)			PERIOD OF FIXATION	SENSITIZED CELLS (ML.)	PERIOD OF HEMOLYSIS
				1	2	6			
Undiluted	0.2	0.1		0.1			4 hr	0.2	15 min
							36 C		37 C
Undiluted	0.2			0.2				0.2	
Undiluted	0.2	0.1			0.1			0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	
1:2			0.1			0.1		0.2	

\*Varies according to range of activity (see Table V)

†Balance to a volume of 0.4 ml according to amount of dilution pipetted

TABLE VIII TYPICAL RESULTS OF TESTS TO DETERMINE THE TITER OF SERUM OF CEREBROSPINAL FLUID

SPECIMEN	DILUTION OF SPECIMEN	AMOUNT PIPETTED (ML.)	AMOUNT TESTED (ML.)	PER CENT HEMOLYSIS WITH UNITS OF COMPLEMENT				VALUE OF (S)* (READ FROM TABLE IX)	VALUE OF (S+A)† (READ FROM TABLE II OF V)	TITER (S+A)† (S)
				1	2	3	6			
Serum	Undiluted	0.05	0.05	25				123		
	Undiluted	0.05	0.05	25				123		
	Undiluted	0.05	0.05		95					
	1:4	0.05	0.0125				0		>36	
	1:5	0.05	0.01				15		39	
	1:6	0.05	0.0083				30		41	
	1:7.5	0.05	0.0067				50		44	44=36
										123
	1:9.5	0.05	0.003				80		42	
	1:12.5	0.05	0.004				100		<50	
Cerebrospinal fluid	Undiluted	0.2	0.2	45				104		
	Undiluted	0.2	0.2		100					
	1:2	0.16	0.08				0		>23	
	1:2	0.13	0.065				5		28	
	1:2	0.1	0.05				25		29	29=28
										104
	1:2	0.08	0.04				60		28	
	1:2	0.07	0.035				80		27	
	1:2	0.06	0.03				90		27	

(S) The reaction with the specimen alone

†(S + A) The reaction with specimen and antigen



TABLE IX UNITS OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS INDICATED BY DEGREES OF HEMOLYSIS OBTAINED WITH DIFFERENT AMOUNTS OF COMPLEMENT IN TESTS WITH SPECIMENS (SERUM OR CEREBROSPINAL FLUID) OR WITH SPECIMENS AND ANTIGEN

UNITS OF COMPLEMENT	PER CENT HEMOLYSIS																	
	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
1	178	154	139	132	123	119	112	109	104	10	096	093	088	085	081	076	071	065
2	351	306	281	263	249	237	226	216	207	20	192	184	176	169	16	152	142	129
3	50	45	41	39	37	35	34	32	31	30	29	28	26	25	24	23	21	19
6	91	83	78	74	72	68	67	64	62	60	58	56	54	52	49	47	44	40
12	150	144	140	135	133	130	127	125	122	120	117	115	112	108	105	101	95	88

The 50 per cent unit values of the reactions in the controls of the specimen without antigen are also read from Table IX. Determine the titer by dividing the maximum value for (S+A), Table II or V by the maximum value for (S) Table IX. Consider specimens anticomplementary if the reaction without antigen is 20 or greater. Anticomplementary reactions can be measured, but since it is doubtful how much this property of serum or cerebrospinal fluid affects the fixation due to syphilitic reagent the titers obtained with anticomplementary specimens must be considered approximate values only. Always include controls (S) with sufficient complement to produce partial degrees of hemolysis in tests of anticomplementary specimens. Whenever the value of (S) is less than 1 the value of (S+A) represents the titer of the specimen that is a value of less than 1 is never used in the denominator of the fraction  $\frac{(S+A)}{(S)}$ .

*Evaluation of Results*—Compare the maximum reaction obtained with serum and antigen in the final titration with that obtained in the titer range

TABLE X CHART TO AID IN DETERMINING RELATIVE DISCREPANCY  
(IF B IS LESS THAN INDICATED, THE RELATIVE DISCREPANCY BETWEEN  
THE NUMBERS (A AND B) IS LESS THAN 25 PER CENT)

A	B	A	B	A	B	A	B
98	126	182	234	336	432	616	792
101.5	130.5	185.5	238.5	343	441	630	810
105	135	189	243	350	450	644	828
108.5	139.5	192.5	247.5	357	459	658	846
112	144	196	252	364	468	672	864
115.5	148.5	203	261	371	477	686	882
119	153	210	270	378	486	700	900
122.5	157.5	217	279	385	495	714	918
126	162	224	288	392	504	728	936
129.5	166.5	231	297	406	522	742	954
133	171	238	306	420	540	756	972
136.5	175.5	245	315	434	558	770	990
140	180	252	324	448	576	784	1008
143.5	184.5	259	333	462	594	812	1044
147	189	266	342	476	612	840	1080
150.5	193.5	273	351	490	630	868	1116
154	198	280	360	504	648	896	1152
157.5	202.5	287	369	518	666	924	1188
161	207	294	378	532	684	952	1224
164.5	211.5	301	387	546	702	980	1260
168	216	308	396	560	720		
171.5	220.5	315	405	574	738		
175	225	322	414	588	756		
178.5	229.5	329	423	602	774		

test. Determine the relative discrepancy either by dividing the difference of the two results by the mean or by comparing them with the pairs of numbers listed in Table X. Repeat the examination of specimens if the findings show more than 25 per cent relative discrepancy. If controls without antigen have been included in the titer range test comparison of the ratios,  $\frac{(S+A)}{(S)}$ , rather than of the values, (S+A) usually shows closer agreement and less repetition is required.

## DISCUSSION

In routine practice in this laboratory about 85 per cent of specimens received for serologic tests for syphilis can be reported as not reacting on the basis of two preliminary tests—one of complement fixation<sup>16b</sup> and the other of precipitation<sup>16c</sup>, both tests are oversensitive and require one tube each. The remaining reacting sera are tested in the six-tube quantitative procedure,<sup>16d</sup> in which titers up to 10 are determined. Specimens having titers greater than 10—about half of those examined quantitatively—are then tested in a two tube titer range test, which indicates the dilutions to be used in determining the exact titer. The successive tests reduce to a minimum the number of specimens requiring titration in dilution and some advantage is derived from the confirmatory evidence they afford.

Studies are being continued to modify the technique of the six tube quantitative test to extend the range of determinable titer somewhat beyond 10. It is hoped that this will largely eliminate, or permit better interpretation of, the so called atypical reaction in these lower ranges and also avoid the inaccurate index of titer occasionally encountered in the tube containing 12 units of complement which now requires a supplementary test to correct. Possibly a greater flexibility of procedure will be provided also.

## SUMMARY

A simplified technique for determining titers between 10 and 100 in the quantitative complement fixation test for syphilis is described. Two amounts of serum or cerebrospinal fluid are tested to determine the range of reactivity and from four to six dilutions are tested to determine the exact titer. Although the method is designed primarily for determination of titers under 100, it is also applicable to determinations of higher titers.

The procedure results in material saving of time and reagents as compared with previous methods.

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# THE PREPARATION OF FROZEN SECTIONS FOR USE IN THE CHEMOSURGICAL TECHNIQUE FOR THE MICROSCOPICALLY CONTROLLED EXCISION OF CANCER

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THE microscopic control of excision which characterizes the chemosurgical technique for the treatment of cancer is attained by the use of frozen sections of specimens removed from the areas under suspicion. As previously described,<sup>1,6</sup> the chemosurgical technique consists of the following steps: (1) the fixation in situ of the tissues by the application of a paste containing a fixative chemical such as zinc chloride; (2) the excision of a layer of fixed tissue which is divided into convenient specimens, the locations of which are mapped on paper and on the lesion; (3) the preparation of frozen sections by cutting through the under surface of each specimen; (4) the scanning of the sections under the microscope so that the remaining areas of cancer can be indicated on the map; (5) reapplication of the fixative to the cancerous parts of the lesion; (6) repetition of the process until the entire area is free of cancer.

This article concerns the microtechnical procedures by which the frozen sections are made. Special consideration of the frozen section technique as applied to the chemosurgical specimen is desirable because of the importance of obtaining complete sections of the specimens which often are rather large and sometimes are friable. The sections must be complete because if any part of a specimen is lost during the sectioning process, the operator cannot know whether the lost tissue was cancerous or noncancerous and therefore cannot know whether further treatment is required. These considerations have necessitated the development of some new details of technique. The usual procedures for the preparation of frozen sections along with the modifications which have been found useful are given herewith.

## TECHNIQUE

*Mounting of the Specimens*—The specimens as they are excised from the lesion are usually flat pieces of tissue which are approximately 1 cm across. Since it is the under surface of these specimens that is to be investigated, the specimen is frozen on the stage by placing it in a small pool of albumin solution\* which has been partially frozen by rapidly opening and closing the valve

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\*Albumin solution is used because when it freezes the ice is not so hard and brittle as when plain water is used. It also holds the specimen on the stage more firmly. It is made by adding approximately 0.5 Gm of albumin to 30 cc of water and preserving with a drop of 95 per cent phenol.

several times. Although the specimen has ordinarily been flattened before placement upon the stage, it is often necessary to elevate one or more edges by cutting into the ice under the specimen with a knife. If the tissue is irregular in thickness it may be necessary to flatten it with the broad surface of a scalpel handle before mounting it on the stage.

It is important that the specimen be oriented so that the microtome knife strikes the narrowest part of the specimen first and moves diagonally over the greatest area (Fig 1, A, B, C, and D). Specimens containing connective tissue, muscle fibers, or cartilage should be mounted so that the fibers (or the long axis of the cartilage) are parallel to the direction of movement of the knife.

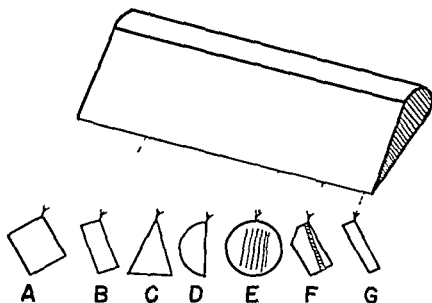


Fig 1—Diagrams showing the recommended angle at which the microtome knife should meet specimens of different shapes and consistencies

(Fig 1, E and F) If a vertical section of the flat piece of tissue is cut its long axis is placed at an angle of about  $65^\circ$  in relation to the knife (Fig 1 G). The specimen should never be placed so that the entire edge meets the knife at the same time because this will crush the tissue, cause the knife to jump over the specimen, or push the entire block off the stage.

Specimens containing skin are placed so that the skin edge is not cut first. Likewise tissues which are heterogeneous in consistency are placed so that the softer part of the tissue is cut first. This provides a firm backing for the knife to cut against.

*Cutting of the Specimen*—When the block of ice has been frozen to the optimum degree of hardness, the section is cut with the microtome knife which is usually used dry and at room temperature. Each section that is cut adheres to the blade and is removed with the moist finger and placed in a glass dish of cold water. If the tissues are fragile this operation must be done gently. If the tissues are pulled apart by the surface tension as they are placed in the water, it is advisable to add a few drops of an aqueous solution of a surface tension depressant such as Zephiran.

Sections are best cut at a thickness of 15 to 25 microns. If the tissues are cut thinner, those that do not contain fairly large amounts of connective

tissue will fall apart. Cancer tissues with a meager connective tissue stroma, fatty tissues, and tissues heavily infiltrated with leucocytes are likely to be fragile, while tissue containing cartilage and considerable amounts of connective tissue will hold together even though cut very thin.

It is particularly important that the knife be kept very sharp. Ordinarily it should be stropped daily and honed weekly. For best results the microtome should be one which is constructed so that the knife is held firmly. A knife-holder which slides on tracks, such as in the Bausch and Lomb clinical microtome is satisfactory from this standpoint.

Ordinarily, four or five complete sections are cut from each specimen. The water in the dish is changed after cutting each specimen so as to avoid mixing of the sections from different specimens.

*Mounting of the Sections*—The sections are worked onto the slide with a curved metal teasing needle. If there is a tendency for the specimen to break up it is important that the fragments be pieced together so that no part of the section is missing. The edges of the specimens usually have been marked with coloring materials (methylomün, washing bluing, and India ink) for the purpose of orientation when the sections are examined under the microscope. These markings which persist throughout the staining process aid in the rearrangement of any fragments which may fall out. Any folded areas may be remounted by floating off that area and remounting without detaching the whole section. If the section is curled up it may be uncurled by attaching one edge to the slide and drawing the slide out of the water so as to unroll it. If the section is not too fragile, it frequently is possible to work the roll loose by gently moving it around in the water with the wire teaser.

It usually is advisable to put three sections on one slide, particularly if they are fragile and if there is some likelihood of the sections being incomplete. However, one good section is all that is necessary. The slide is then blotted carefully and thoroughly with a very dry soft towel, preferably an old one that has the lint washed from it. It is best to blot only one of the sections first to see if the section will cling to the towel. If it does so, the other sections may be fixed to the slide by passing it through an alcohol flame several times. The slide is numbered with a wax pencil and put in 95 per cent alcohol to await staining.

*Staining*—The various solutions used in the staining process are kept in Coplin jars. These jars contain five slots so it is convenient to run five slides at a time, keeping slide number one in slot number one, slide number two in slot number two, and so forth. Some of the slides may be run ahead as long as they are kept in their proper slot so that there is no danger of getting the sections mixed.

The procedure for the dehydration, staining, clearing, and mounting of the sections is as follows:

1 *Ninety-five per cent alcohol* The slide may be left in this solution for any time over fifteen seconds.

2 *Cellulose nitrate (Parlodion\*) solution* The slide is quickly dipped

\*Mallinckrodt Chemical Works, St. Louis, Mo.

in this solution which should be rather thin. If it is too thick it and the sections tend to peel off the slide. Moreover a thick layer prevents the ready penetration of the stain and prolongs the process. The solution is made by dissolving one stick (1 Gm.) of paralodion in 30 cc. of a solution of equal parts of ether and 95 per cent alcohol. After dipping in the cellulose nitrate solution the slide is dried by immediately blotting firmly with a clean dry towel.

3 *Hematoxylin* Approximately two minutes is the usual staining time but the slide may be left in longer if the tissue does not take the stain well. Delafield's hematoxylin is used. This stain lasts indefinitely but it is advisable to filter off the precipitate every day before using. After staining the excess dye is washed off the slide in a dish of water.

4 *Acid alcohol* This solution is just strong enough to destain the average section with one dip. It is made by adding 25 cc. of concentrated hydrochloric acid to 500 cc. of 70 per cent ethanol. This solution is changed once a week or more often if necessary.

5 *Alkaline water* If the tap water is alkaline it is convenient to let the slide stand in a dish of water to alkalize. Otherwise a weak solution of sodium bicarbonate is used and the sections are allowed to remain in the solution until they are blue. This usually takes one or two minutes. Only one slide should be in the solution at a time because the sections may loosen and come off at this time and be mixed. If a section floats off it may be mounted as before, blotted with a dry towel, and continued in the staining process. It is advisable to blot the sections at this stage to insure the removal of water and bubbles from under the sections.

6 *Eosin* The solution is adjusted so that one dip of the section gives adequate staining. The stock solution is made as follows. Dissolve 2.5 Gm. aqueous eosin in 500 cc. distilled water. Add 4 cc. of concentrated hydrochloric acid and 1 cc. of glacial acetic acid. Pour off the supernatant and save the precipitate which is washed six times with water. Filter and dry the precipitate. There will be about 0.5 Gm. of precipitate. This is dissolved in 100 cc. of 95 per cent alcohol. The stain may be diluted with 95 per cent alcohol if it stains too deeply. Alcohol is also added as needed to replace that lost by evaporation. The solution should be filtered whenever particulate matter gathers in it.

7 *Alcohol and ether* Treat with this solution for two minutes or more. This solution is made up of equal parts of ether and 95 per cent alcohol and it should be changed daily.

8 *Absolute alcohol* Treat with this solution for one to two minutes or until all of the paralodion is removed. This solution should be changed once a week or more often if necessary for complete dehydration of the sections.

9 *Carbol xylol* Treat with this solution until the tissues are clear. Two minutes or more may be required. The stock solution is made by adding 300 cc. of xylol to 100 Gm. of phenol crystals. The solution ordinarily is changed once a week.



10 *Xylol* Treat with this for two minutes or more Change the solution weekly

11 *Mount in Clarite* and cover with a 22 by 50 mm cover slip This large size cover slip is desirable because several sections are placed on each slide The clarite solution is made by dissolving 60 Gm of clarite "X" hydrocarbon resin in 40 cc of toluene Clarite is preferable to balsam because it hardens more rapidly and because it does not become yellow with age

Sections made with this technique have not deteriorated after periods of over twelve years

### SUMMARY

The microscopic control of excision attained by the chemosurgical treatment of cancer is dependent upon the completeness of the frozen sections of the tissues which have been fixed in situ prior to excision To make possible these complete sections a number of modifications of the usual frozen section technique have been devised A procedure embodying these modifications is described The sections, which are stained with hematoxylin and eosin, are permanent

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### Erratum

In the paper by Dominguez, Corcoran, and Page, *Mannitol Kinetics of Distribution, Excretion, and Utilization in Human Beings*, which appeared in the October issue of the *JOURNAL* (32 1192, 1947) equation 13 of Table I should be

$$V = \frac{A_2 (\beta^2 a + a^2 b)}{\alpha \beta (\beta a + ab)}$$

or, in terms of G,

$$V_2 = \frac{G (\beta^2 a + a^2 b)}{(\beta a + ab)^2}$$

# PTEROYLGLUTAMIC ACID DEFICIENCY IN SWINE EFFECTS OF TREATMENT WITH PTEROYLGLUTAMIC ACID LIVER EXTRACT AND PROTEIN

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THE discovery of the effectiveness of pteroylglutamic acid in the treatment of the macrocytic anemias has, so far, failed to clarify our understanding of the pathogenesis of these anemias.<sup>1</sup> It has now been demonstrated that neither pteroylglutamic acid nor its naturally occurring conjugate the heptaglutamate, is the extrinsic factor the intrinsic factor or the potent antipernicious anemia substance in purified liver extract. Furthermore it has become increasingly evident that pteroylglutamic acid neither corrects nor prevents the neurological manifestations of pernicious anemia.<sup>2</sup> With regard to the role of pteroylglutamic acid in pernicious anemia it has been suggested that in some patients with this disorder there is in addition to other metabolic defects an inability to utilize the naturally occurring pteroylheptaglutamate.<sup>1</sup> It has also been proposed that pteroylglutamic acid and the antipernicious anemia principle of liver may function via unrelated pathways.<sup>1</sup>

The purpose of this paper is to describe the hematologic manifestations of pteroylglutamic acid deficiency in swine produced by the administration of a synthetic substance having an action antagonistic to that of pteroylglutamic acid and to compare the therapeutic effectiveness of purified liver extract and synthetic pteroylglutamic acid in this condition. This report leaves many questions unanswered and therefore must be considered preliminary in nature. The observations are now being amplified and extended.

The pteroylglutamic acid antagonist used in these experiments (N67†) was prepared‡ by allowing 2, 4, 5 triamino 6 hydroxypyrimidine and p amino benzoyl 1(+) glutamic acid to react with 2, 3 dibromobutyraldehyde. Since the reaction product (N67) has not been purified it has been termed crude methyl folic acid. The product of the reaction described has been designated by Martin, Tolman and Moss<sup>3</sup> as 7 methylfolic acid. The substance antagonizes reversibly the effect of pteroylglutamic acid on the growth of *Streptococcus faecalis* R and of *Lactobacillus casei*.<sup>4</sup> Different inhibiting ratios have been reported for the two organisms. The ratio of antagonist to pteroylglutamic acid for *Str faecalis* R is 20:1 and for *L casei* about 1000:1.

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Fellow of the United States Public Health Service.

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‡By Dr. M. L. Hultquist and Dr. J. M. Smith, Jr. of the Calco Chemical Division, American Cyanamid Company, Pearl River, N. Y.

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Sections made with this technique have not deteriorated after periods of over twelve years

(milligrams per kilogram body weight daily) thiamin hydrochloride 0.25 riboflavin, 0.12, nicotinic acid 1.20, pyridoxine hydrochloride 0.20 calcium pantothenate 0.50, choline chloride 50.00 para aminobenzoic acid 0.10 inositol 0.10 pteroylglutamic acid 0.30. In addition all animals received crystalline biotin 50  $\mu$  per kilogram body weight per week intramuscularly. Sulfasuxidine when given was added to the basal diet in amounts of 2.0 per cent. The antagonist when given was administered daily in capsules in amounts of 0.2 per cent of the basal diet (equivalent to 0.06 gm per kilogram body weight). Full details of the experimental methods have been published elsewhere.<sup>1</sup>

The four animals receiving the antagonist were first depleted of pteroylglutamic acid for periods of eighty six to one hundred twenty two days by a diet lacking pteroylglutamic acid supplemented with sulfasuxidine.

Plasma iron determinations were made by the method of Bailan and Walker.<sup>12</sup> Serum copper was measured by the method of Critchfield, Jones and Wintrobe.<sup>1</sup> For the determination of free erythrocyte protoporphyrin the method of Ginstem and Witson<sup>13</sup> was used.

Specimens of bone marrow were obtained by aspiration of the sternal marrow with standard 16 gauge sternal puncture needles. A small amount of marrow fluid usually less than 0.3 ml. was withdrawn into a clean dry syringe and thin cover glass preparations were drawn and stained with Wright's stain. From 500 to 1500 cells were counted in each preparation. The figures are expressed in per cent of total cells counted.

In order to determine whether or not extrinsic factor is present in the crude casein used in these studies the material was assayed in three patients with classic uncomplicated Addisonian pernicious anemia. The procedure of assay was as follows. The patients were hospitalized and during the assay periods liver meat meat products milk and poultry were excluded from the diet. Bread cereals sugar fats vegetables and fruits were permitted in the amounts desired. Fifty gram of the casein to be assayed were incubated at 37° C. for two hours with 150 to 200 ml. of normal human gastric juice at pH 2.5 to 3.5. The incubation mixture was then strained through cheesecloth the filtrate was neutralized to pH 5 and administered immediately to the patient. The results

TABLE I. ASSAY OF CRUDE CASEIN FOR EXTRINSIC FACTOR ACTIVITY

PATIENT	PERIOD		TREATMENT		MAX. EFTICS (%)	VOLUME PACKED RED CELLS (CC/100 CC)	
	No.	DURATION (DAYS)	TYPE	DURATION (DAYS)		BEGIN	END
T. S.	I	8	None	8	1.0	27.8	24.2
	II	20	Crude casein + alcoholic extract	10	9.4	24.2	34.0
	III	12	Liver extract 1 u/day I.M.	10	1.8	34.0	39.0
N. B.	I	12	None	12	4.4	23.4	23.8
	II	12	Extracted casein + 3rd alcoholic extract	10	12.8	23.8	29.0
	III	10	Liver extract 1 u/day I.M.	10	2.6	29.0	33.8
S. S.	I	22	None	22	2.2	26.4	27.0
	II	16	Crude casein	10	9.0	27.0	31.8
	III	10	Liver extract 1 u/day I.M.	10	0.8	31.8	32.0

of the assays are presented in Table I. Patient J S was given, in addition to the filtrate, an alcoholic extract from crude casein. This was prepared by adding 18 liters of 65 per cent ethanol to 6 kg of casein. The casein-alcohol mixture was allowed to stand for forty-eight hours at room temperature with intermittent shaking. The alcohol was then filtered off and distilled in vacuo to dryness. The residue was extracted with several volumes of ethyl ether to remove the fats, and dried at room temperature, and then pulverized. One tenth of the resulting residue was added daily to the crude casein prior to incubation.

As seen in Table I following the administration of the described mixture to Patient J S a reticulocytosis of 8.4 per cent developed and in twenty days the volume of packed red cells increased from 24.2 ml per 100 ml to 34.0. One unit of purified liver extract (15 units per milliliter) was then administered intramuscularly daily for ten days. No secondary reticulocyte response occurred. Patient N B was given crude casein, extracted three times as described plus the residue from the third alcohol extraction. As can be seen, the extraction procedure failed to remove the extrinsic factor activity and a reticulocytosis developed which reached a maximum of 12.8 per cent. One unit of liver extract daily again failed to elicit a secondary reticulocytosis. Patient S S was given 50 Gm of the crude casein daily without the addition of an alcoholic extract. A reticulocytosis of 9.0 per cent developed and the volume of packed red cells rose from 27.0 ml per 100 ml to 31.8 in sixteen days. Following this, the daily intramuscular injection of 1 unit of purified liver extract failed to produce a secondary reticulocytosis.

Since the administration of a total of 500 Gm of crude casein caused a significant response in Patient S S and since the animals received approximately 15 to 120 Gm of crude casein daily for about 190 days, it must be concluded that our experimental animals were receiving substantial amounts of extrinsic factor. It is unlikely that the response in the patients with pernicious anemia was due to the pteroylglutamic acid content of the crude casein since the total pteroylglutamic acid content of the casein, after enzymatic digestion as determined titrimetrically with *L. casei* by the method of Teph and Elvehjem<sup>14</sup> was found to be only 0.009  $\mu$ g per 100 Gm of casein. The alcohol extracted casein was found to contain only 0.006  $\mu$ g per cent by this method.\*

## RESULTS

*General*—The animals receiving the pteroylglutamic acid antagonist became listless and weak and ate poorly. Hair loss was not extensive but the hair became thin and lusterless. The general appearance of the animals was extremely untidy. The abdominal walls of the pigs sagged, probably due to loss of muscle tone. As the deficiency progressed, then squeals became faint and weak.

A moderately severe diarrhea was present, the stools being somewhat orange-yellow in color due presumably to the presence of antagonist. Growth was poor, and about equal, in both the control and antagonist groups due to the low content of protein (10 per cent) in the diet. Oral lesions were not observed.

\*We are indebted to Dr J M Cooperman, Hoffmann-La Roche Inc., Nutley, N. J. for these determinations.

*Peripheral Blood*—In contrast to the control animals those receiving the antagonist became markedly anemic in twenty one to forty two days. The volume of packed red cells decreased from a level of about 35 ml to approximately 20 ml per 100 ml (Table II Figs 1-4). The anemia at this time was normocytic. Examination of the blood smears revealed marked anisocytosis without a significant degree of poikilocytosis, an increase in Howell Jolly bodies, frequent nucleated red blood cells, and moderate polychromatophilia. Unusually large macrocytes were frequently seen but microcytes were equally numerous. This is illustrated in Fig. 6.

TABLE II DATA ON ANEMIA AND RED BLOOD CELL MORPHOLOGY IN LOW PROTEIN CONTROL GROUP AND PCA ANTAGONIST GROUP

	LOW PROTEIN CONTROL					MEAN	PCA ANTAGONIST					MEAN
Pig	10 45	10 46	10 47	10 48	10 50		10 53	10 54	10 56	10 64		
Days on experiment	156	156	130	156	156	151	164	113	105	146	132	
Days on antagonist	0	0	0	0	0	0	42	25	21	36	31	
RBC $\times 10^6$ (cmm)	6.96	6.36	5.59	6.35	6.51	6.33	3.53	3.16	2.76	4.03	3.37	
Hb (Gm %)	12.0	11.8	10.3	12.0	11.3	11.5	8.1	5.7	5.6	6.7	6.5	
Ht (cc/100 cc)	36.0	35.2	30.0	36.4	34.4	34.4	24.4	18.0	15.0	20.8	19.5	
MCV ( $\mu$ )	52	55	54	57	53	54	69	57	54	52	58	
MCH ( $\gamma$ )	17	19	18	19	17	18	23	18	18	17	19	
MCHC (%)	33	33	34	33	33	33	33	32	34	32	33	
Reties (%)	1.0	2.6	2.0	2.0	0.6	1.6	4.0	4.4	2.6	1.2	3.0	

Ht Volume of packed red cells MCV mean corpuscular volume MCH mean corpuscular hemoglobin MCHC mean corpuscular hemoglobin concentration

As shown in Table III the animals receiving the antagonist developed a mild leucopenia and severe neutropenia. The mean total leucocyte count for the control group was 15 000 per cubic millimeter, as compared with 10 500 for the pigs receiving the antagonist. The percentage of granulocytes (metamyelocytes, neutrophils, basophils and eosinophils) was reduced in the antagonist group to 14 as compared with a mean of 47 for the control group. The absolute number of granulocytes in the control animals averaged 7 000 per cubic millimeter of blood, whereas in the animals receiving the antagonist there were only 1 500 per cubic millimeter. Giant metamyelocytes and multinucleated neutrophils such as are found in the blood smears of patients with pernicious anemia were not seen in the blood of the pigs.

The number of platelets per cubic millimeter of blood in the control animals ranged from 310,000 to 510 000. The number of platelets per cubic millimeter of blood in the animals receiving antagonist was extremely variable. Values between 100,000 and 310,000 were frequently obtained but in one animal (10 64) the number of platelets increased prior to therapy.

*Bone Marrow*—In Table IV the results of differential cell counts on sternal marrow obtained from the eight control animals and the four animals receiving antagonist are summarized. In the marrows from the animals receiving antagonist there was a marked reduction in the number of polymorphonuclear neutrophils and neutrophilic metamyelocytes as well as a slight increase in earlier forms of the myeloid series and a significant decrease in the leucocyte erythroid ratio. In addition extremely immature and somewhat abnormal nucleated red cells were present (Fig. 7). These cells were round or oval and extremely large measuring 12 to 25  $\mu$  in diameter, whereas the most immature nucleated

TABLE III DATA ON LYUCOCYTES AND PLATELETS IN LOW PROTEIN (CONTROL GROUP) AND PGA ANTAGONIST GROUP

	Mean Range	LOW PROTEIN CONTROL						MEAN	PGA ANTAGONIST						MEAN
		10.45	10.46	10.47	10.48	10.50	10.53		10.54	10.56	10.64				
Pig WBC $\times 1000$ (cmm)		15.2	15.6	13.4	16.4	14.6	13.0	8.4	11.9	8.2	13.6	10.5			
		14.1	16.2	14.3	17.6	12.7	14.1	12.7	7.7	8.8	6.4	10.2	9.4	17.1	6.4
PMN (%)		44	50	48	48	47	47	8	18	16*	14	14			
		41.48	42.53	45.51	40.53	37.50	37.55	1.13	8.23	13.19	10.19	1.23			
MON (%)		56	50	52	52	56	53	92	92	84*	86	86			
		52.59	47.58	49.55	45.60	51.63	45.63	87.99	77.92	81.87	81.90	77.99			
PMN $\times 1000$ (cmm)		6.7	7.8	6.4	7.9	6.4	7.0	0.7	2.1	1.3*	1.9	1.5			
		5.9	7.5	6.9	6.7	7.0	5.7	0.1	0.6	0.3	0.9	0.1	0.3	0.1	0.3
NRBC per 100 WBC		1	0	3	0	2	1	11	11	5*	1	7			
		0.3	0.1	1.4	0	0.3	0.4	7.15	7.14	5.6	0.1	0.15			
Platelets $\times 1000$ (cmm)		460	470	350	410	380	414	307	300*	415	415	341			
		420	510	450	480	430	510	100	680	220	630	100	680		

The means represent an average of three separate determinations

PMN Polymorphonuclear cells including basophils and eosinophils MON mononuclear cells including both lymphocytes and monocytes NRBC nucleated red blood cells

\*Two determinations only

TABLE IV SUMMARY OF BONE MARROW STUDIES ON EIGHT LOW PROTEIN CONTROL ANIMALS AND FOUR ANIMALS RECEIVING PCA ANTAGONIST

CELL TYPE	CONTROL		PCA ANTAGONIST	
	MEAN	RANGE	MEAN	RANGE
Megakaryocyte	0.9	0.4-1.3	2.0	0.4-3.4
Promegakaryocyte	1.6	0.3-3.8	4.4	2.0-5.8
N Megakaryocyte	6.8	2.6-10.0	7.2	4.4-12.4
E Megakaryocyte	0.8	0.3-1.4	2.4	1.2-2.8
N Metamegakaryocyte	16.6	2.8-46.8	18.0	13.4-25.6
E Metamegakaryocyte	0.3	0.0-1.0	1.0	0.0-1.8
PMN Neutrophil	12.4	3-20.6	3.8	1-7.0
PMN Eosinophil	0.2	0.0-0.6	0.3	0.0-0.6
Lymphocyte	7.0	4.3-9.3	3.3	2.0-5.6
Plasma cell	0.1	0.0-0.4	0.1	0.0-0.4
Monocyte	0.3	0.0-0.9	0.2	0.0-0.6
Reticulum cell	0.1	0.0-0.2	1.1	0.0-1.8
Mitotic cell	0.4	0.0-0.9	1.4	0.6-1.8
Megakaryoblast	0.0	0.0	1.3	1.1-2.2
Pronormoblast	0.4	0.0-1.4	2.2	0.8-4.2
B Normoblast	1.7	0.2-3.3	4.7	3.2-6.4
I Normoblast	8.9	2.1-38.3	29.8	19.6-45.2
O Normoblast	1.3	0.0-6	0.8	0.2-1.6
Leucocyte/erythrocyte	2.6	1.5-3.3	0.8	0.6-1.3

N, Neutrophilic; E, eosinophilic; PMN, polymorphonuclear; B, basophilic; P, polychromatophilic; O, orthochromatic

red cells seen in the marrow of the control animals measured 6 to 12 microns. These large cells possessed a relatively large nucleus and a somewhat homogeneous basophilic cytoplasm. The nuclei were composed of rather thin mesh-like strands of chromatin. In some cells the chromatin showed some tendency to clump, in others the chromatin appeared finely granular and more homogeneous. The more immature of these cells contained two or three distinct nucleoli. A delicate nuclear membrane surrounded the nucleus. Later stages of this cell were present including the orthochromatic stage.

These cells resembled closely the megakaryoblastic series of cells seen in the marrow of patients with pernicious anemia, the only distinct difference being that the nuclear chromatin was not so fine and meshlike as that seen in human bone marrow. Whether or not the cells described as pig megakaryoblasts is a matter for conjecture. In any event similar cells were not seen in the marrow of the control animals nor have they been observed by us in the marrow of animals following severe and prolonged hemorrhage superimposed on a dietary restriction of iron. For purposes of discussion these cells will be referred to as megakaryoblasts in this paper in order to distinguish them from cells of the normoblast series. On several occasions orthochromatic megakaryoblasts were seen in the peripheral blood of the deficient animals.

*Plasma Iron, Serum Copper, and Erythrocyte Protoporphyrin*—Plasma iron, serum copper, and erythrocyte protoporphyrin determinations are presented in Table V. These were made at the time of maximal anemia and prior to the initiation of therapy. No significant difference was noted in either the plasma iron or the erythrocyte protoporphyrin values in the two groups, nor were these determinations appreciably different from those obtained in swine maintained on a diet containing 26 per cent casein.<sup>1</sup> Animals maintained on a low protein diet have been found to have a significantly low serum copper.\*



The serum copper in two of the animals receiving antagonist (10 53 and 10 64) was low, whereas in the other two (10-54 and 10 56) the values were comparable with those obtained in animals on a 26 per cent casein diet<sup>12</sup> The significance of this finding is not obvious

TABLE V STUDIES ON PLASMA IRON, SFRUM COPPEP, AND FREE ERITHROCYTL PROTOPORPHYRIN

GROUP	ANIMAL	PI ( $\mu$ g %)	S CU ( $\mu$ g %)	EP ( $\mu$ g %)
PGA Antagonist	10 53	123	127	79
	10 54	154	220	97
	10 56	118	235	98
	10 64	110	177	100
	Mean	126	190	94
Control	Mean	101	146	108
	Range	66 140	127 179	78 129

PI Plasma iron S Cu serum copper EP erythrocyte protoporphyrin in micrograms per 100 ml red cells

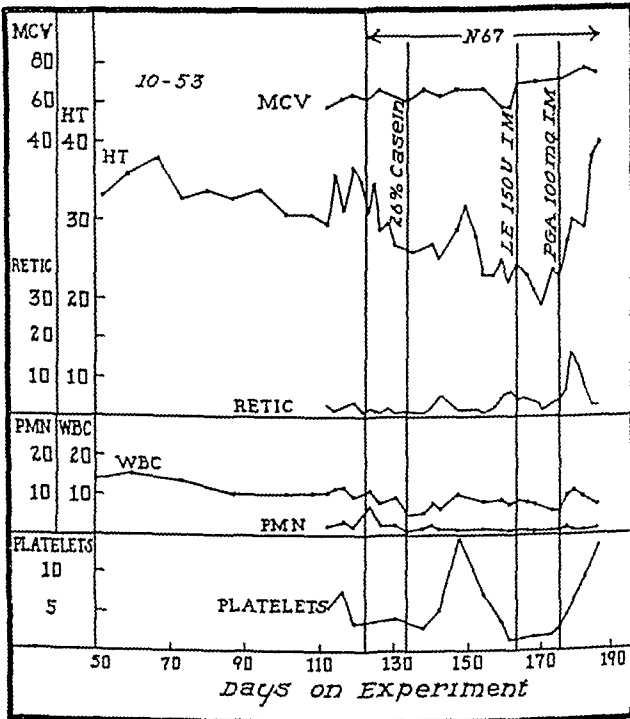


Fig 1—Anemia, leucopenia and neutropenia in a pig (10 53) receiving a pteroyl glutamic acid antagonist (N67). There was no hemopoietic response to the administration of casein or purified liver extract (LE) but a good response occurred when pteroylglutamic acid (PGA) was given.

MCV Mean corpuscular volume in cubic microns. Ht. volume of packed red cells in ml/100 ml. Retic. reticulocytes expressed in per cent. WBC total leucocyte count in thousands per cmm. PMN number of polymorphonuclear cells (mature neutrophils, basophils and eosinophils and metamyelocytes) in thousands per cmm of blood. Platelets expressed in hundred thousands per cmm of blood. IM intramuscular injection. The purified liver extract used contained 15 USP units per milliliter.

*Effect of Therapy on the Peripheral Blood*—One animal (10 53, Fig 1) was treated twelve days after the administration of antagonist was started by increasing the casein in the diet to 26 per cent. There was a temporary increase

in the volume of packed red cells but this increase was not sustained and the animal became severely anemic. There was no apparent increase in the total leucocyte or neutrophil counts and there was no significant reticulocytosis. The platelets increased from a low normal value of 300 000 per cubic millimeter to a value considerably above the normal (1 400 000) and then decreased to the thrombocytopenic level of 100 000. The protein therapy had no apparent effect on the mean corpuscular volume. There was however a significant growth response (Fig 5) and a rise in serum albumin from 2.1 Gm per cent to 3.2 Gm per cent.

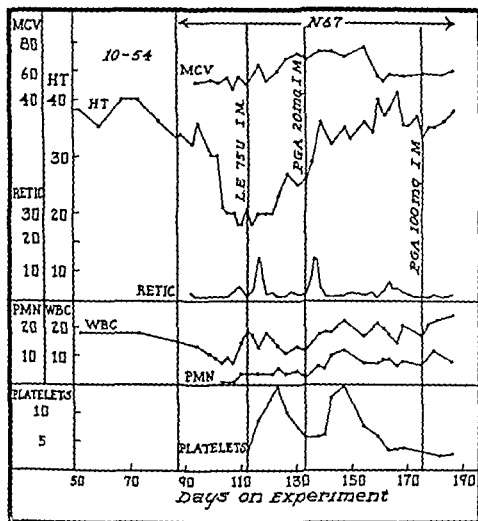


Fig. —Development of anemia, leucopenia and neutropenia in a pig (1054) receiving a pteroylglutamic acid antagonist (N67) and response to purified liver extract (L.E.) and pteroylglutamic acid (PGA). For explanation see Fig. 1.

Three animals (1053, 1054, 1056; Figs. 1, 2, 3) were treated with purified liver extract prior to pteroylglutamic acid therapy. In one (1053, Fig. 1) there was no significant effect on any of the blood constituents following the injection of 150 units. In the other two (1054, 1056, Figs. 2 and 3) there was a suboptimal response consisting of a reticulocytosis of 15 per cent in one animal (1054, Fig. 2) and a modest rise in volume of packed red cells and platelets in both. In one pig (1054, Fig. 2) the total leucocyte count had risen to normal at the time liver therapy was begun. There was no apparent effect from the liver therapy and neutropenia developed again. In the other animal (1056, Fig. 3) the total leucocyte and neutrophil values rose to normal but were not sustained.

These three animals (10-53, 10-54, 10-56, Figs 1-3) were then treated with synthetic pteroylglutamic acid and an immediate, rapid, and maximal increase in the volume of packed red cells followed. In two of the pigs (10-53, 10-54, Figs 1 and 2) a significant reticulocytosis of 16 and 15 per cent, respectively, appeared. An initial rise in the platelet count was observed in two of the animals (10-53, 10-54, Figs 1 and 2), but in the three (10-54, 10-56, 10-64, Figs 2-4) in which the platelets were followed for a significant period of time

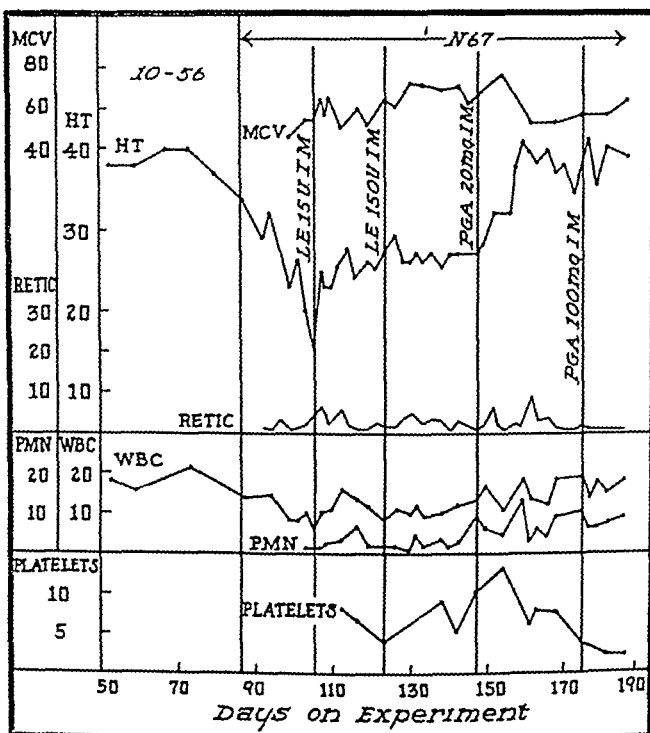


Fig. 3.—Anemia, leucopenia and neutropenia in a pig (10-56) receiving a pteroylglutamic acid antagonist (N67). There was a questionable response to purified liver extract (LE) and a well-marked response to pteroylglutamic acid (PGA). For symbols see Fig. 1.

thrombocytopenia reappeared. In two of the pigs (10-54, 10-56, Figs 2 and 3) the leucopenia and neutropenia disappeared following pteroylglutamic acid therapy. In the third (10-53, Fig. 1), this effect was not observed and only a slight, transient increase in the total leucocyte count was noted.

All three of the pigs treated initially with liver (Figs 1-3) developed definite but not marked macrocytosis following liver therapy (Fig. 6). The mean value for the mean corpuscular volume in the control group (twenty-four determinations in five animals) was  $56 \pm 5.16$  cubic microns with a range of 48 to 69 cubic microns. Only twice were the values above 59 cubic microns. In all three of the pigs treated with liver the mean corpuscular volumes reached 72 cubic microns. The macrocytosis could not be attributed to the presence of reticulocytes since these were not significantly increased. In the pigs (Figs 2 and 3) in which the mean corpuscular volume was followed for a significant

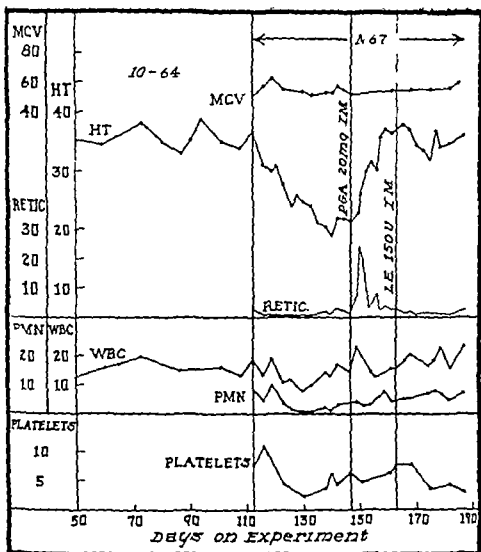


Fig 4—Anemia leucopenia and neutropenia in a pig (10 64) receiving a pteroyl glutamic acid antagonist (N67). There was a pronounced response to pteroyl glutamic acid (PGA) but purified liver extract (LE) had no effect. For explanation see Fig 1

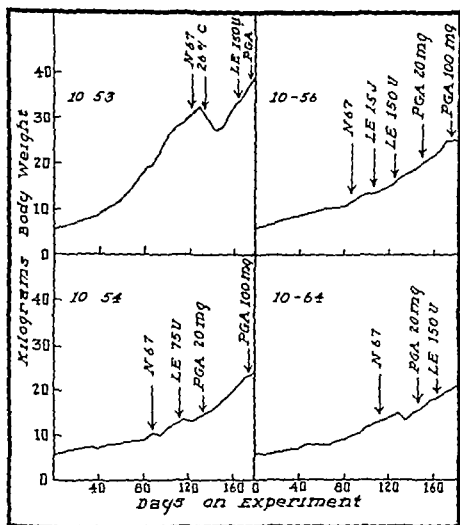


Fig 5—Growth curves of the four animals receiving the pteroyl glutamic acid antagonist (N67)

period after pteroylglutamic acid therapy the macrocytosis disappeared (Fig 6). Likewise the anisocytosis and polychromatophilia disappeared, and the Howell-Jolly bodies and nucleated red cells diminished in numbers. This was not the case following liver therapy. As already noted, macrocytosis developed and, in addition, nucleated red cells, Howell-Jolly bodies, and polychromatophilic cells persisted in increased numbers.

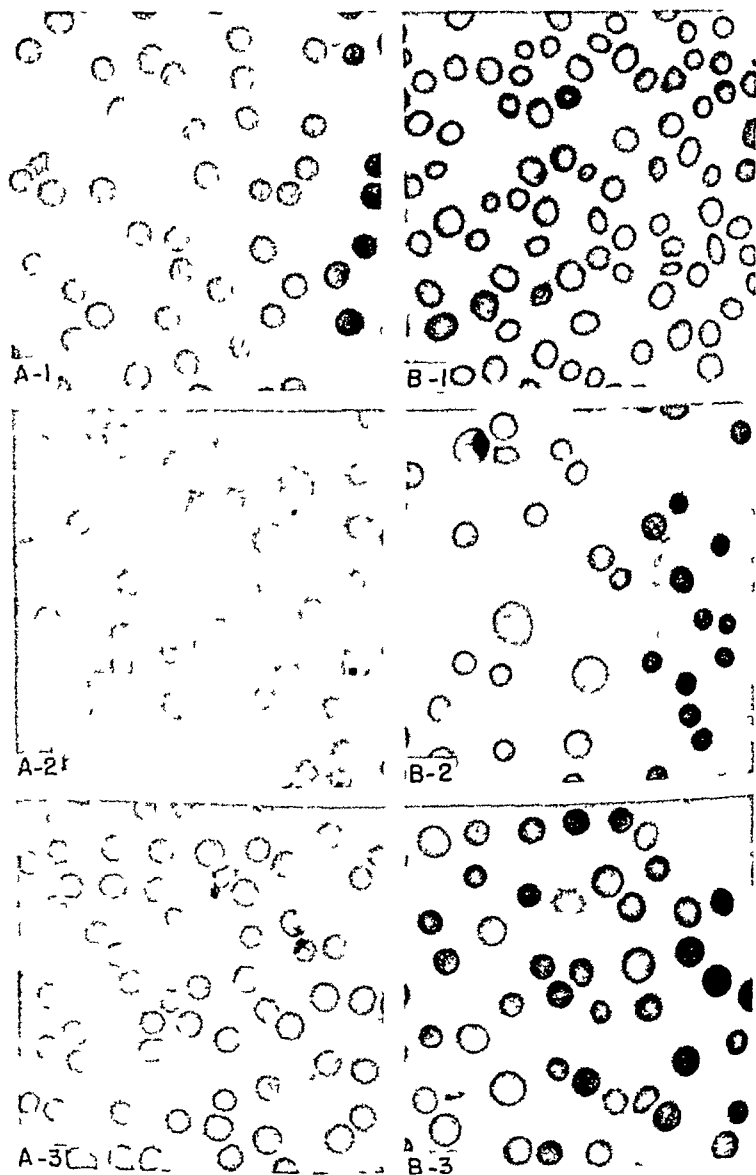


Fig 6—Photomicrographs of peripheral blood smears ( $\times 1000$ ). A-1 Normal pig (10.00) mean corpuscular volume (MCV) 57 cubic microns. A-2 Pig (10-56) receiving PGA antagonist (139th day of experiment) the smear was made after liver extract had been given and prior to PGA therapy, note marked anisocytosis and numerous macrocytes. MCV 69 cubic microns. A-3 From same animal as in A-2 after PGA therapy (183rd day), note decrease in anisocytosis and absence of macrocytes. MCV 57 cubic microns. B-1 Pig (10.00) prior to administration of antagonist (120th day) MCV 64 cubic microns. B-2 From same pig as in B-1 but after the administration of antagonist (164th day) note marked anisocytosis and numerous macrocytes. MCV 69 cubic microns. B-3 From same pig as in B-2 after liver therapy (187th day) note macrocytosis. MCV 72 cubic microns.

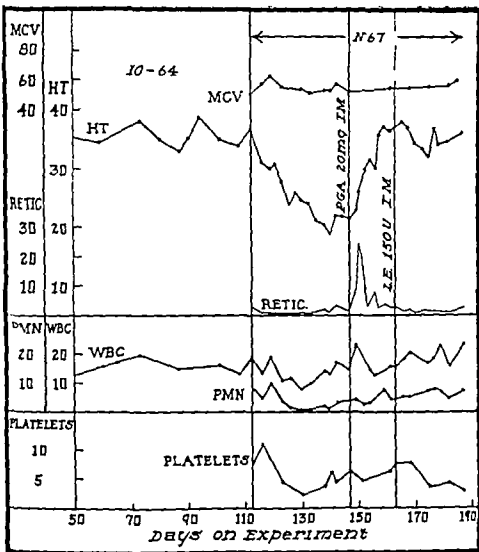


Fig 4—Anemia, leucopenia and neutropenia in a pig (10-64) receiving a pteroyl glutamic acid antagonist (N67). There was a pronounced response to pteroyl glutamic acid (PGA) but purified liver extract (LE) had no effect. For explanation see Fig 1.

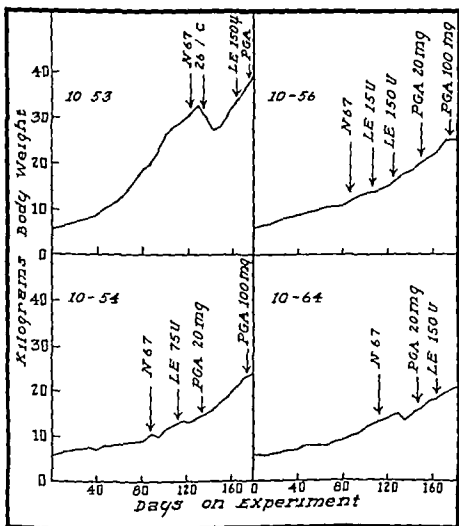


Fig 5—Growth curves of the four animals receiving the pteroyl glutamic acid antagonist (N67).

period after pteroylglutamic acid therapy the macrocytosis disappeared (Fig 6). Likewise the anisocytosis and polychromatophilia disappeared, and the Howell-Jolly bodies and nucleated red cells diminished in numbers. This was not the case following liver therapy. As already noted, macrocytosis developed and, in addition, nucleated red cells, Howell-Jolly bodies, and polychromatophilic cells persisted in increased numbers.

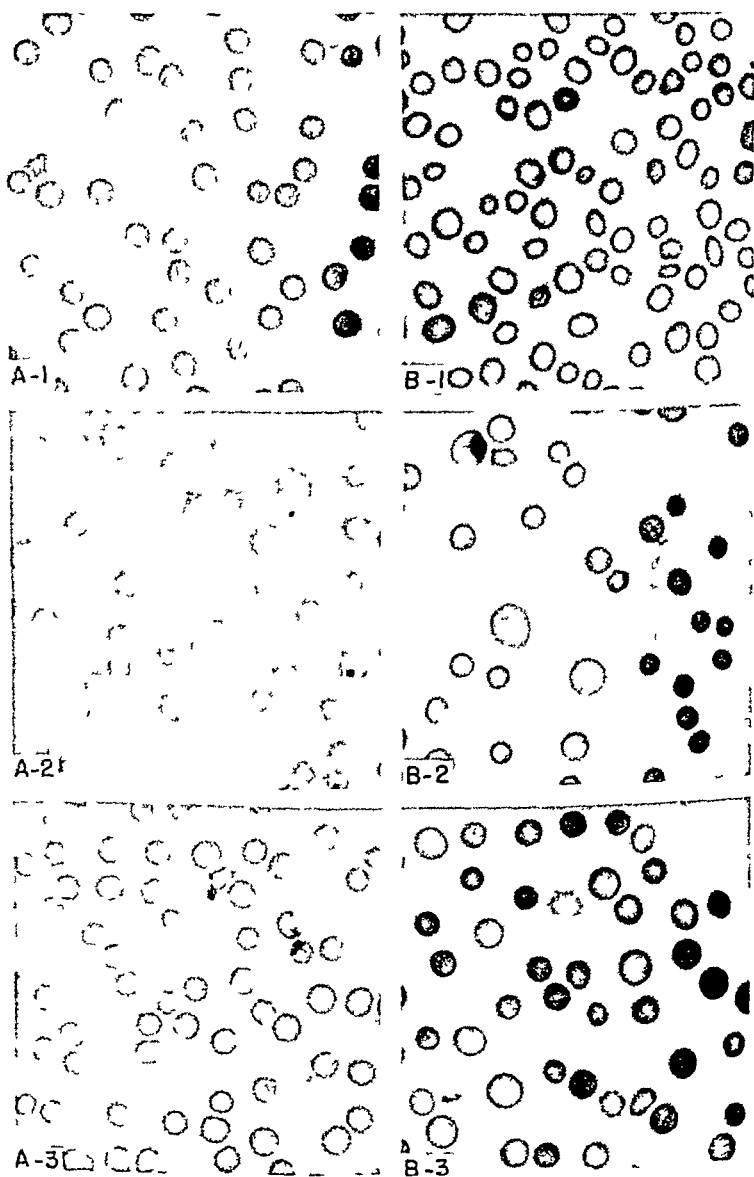


Fig 6—Photomicrographs of peripheral blood smears ( $\times 1000$ ). A-1 Normal pig (10.50) mean corpuscular volume (MCV) 57 cubic microns. A-2 Pig (10.56) receiving PGA antagonist (139th day of experiment) the smear was made after liver extract had been given and prior to PGA therapy. note marked anisocytosis and numerous macrocytes. MCV 69 cubic microns. A-3 From same animal as in A-2 after PGA therapy (183rd day) note decrease in anisocytosis and absence of macrocytes. MCV 57 cubic microns. B-1 Pig (10.51) prior to administration of antagonist (120th day) MCV 64 cubic microns. B-2 From same pig as in B-1 but after the administration of antagonist (164th day) note marked anisocytosis and numerous macrocytes. MCV 69 cubic microns. B-3 From same pig as in B-2 after liver therapy (187th day) note macrocytosis. MCV 72 cubic microns.

One pig (10 64, Fig 4) was treated initially with 20 mg of pteroylglutamic acid. This was followed by an immediate reticulocytosis which reached a maximum of 25 per cent on the third day. At the same time there was a rapid

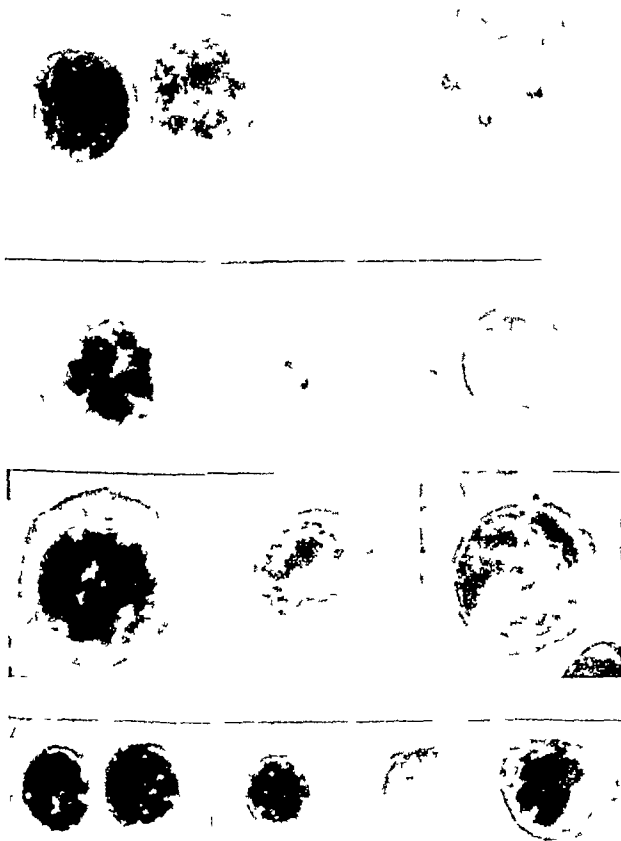


Fig. —Photomicrographs of nucleated red cells ( $\times 900$ ) from the bone marrow of pigs receiving PGA antagonist and of control animals. The cells in the upper three rows are representative of the type of cells seen in the marrows of the animals receiving PGA antagonist and are the cells described in the text as megaloblasts. The cell in the bottom row was taken from the marrows of control pig. Note the difference in the size of the cells and in the character of the nuclear chromatin.



TABLE VI BONE MARROW STUDIES ON ANIMALS RECEIVING PGA ANTAGONIST

Fig	Days after antagonist	10 53				10 54				10 56				10 64			
		0	37	53	64	22	41	45	74	98	21	39	63	74	0	25	35
				LE	PGA		LE		PGA	PGA	LF	LE	PGA				PGA
Therapy				150	100		75		20	100		15	150	20			20
Days after therapy				12	11		17	21	29	11		18	24	15			15
Myeloblast		0 2	2 2	0 9	1 4	3 4	1 2	0 4	0 2	0 2	0 4	1 4	0 8	1 0	1 8	1 6	2 0
Promyelocyte		4 1	5 6	2 3	3 2	5 8	1 8	1 0	3 0	0 8	2 0	3 5	4 2	4 8	2 6	1 5	4 0
N Myelocyte		4 2	4 4	1 7	1 9	12 4	5 0	1 4	4 8	2 2	4 4	5 2	9 0	1 8	4 8	10 5	7 6
L Myelocyte		1 2	2 8	0 7	0 4	2 8	2 2	2 6	1 0	0 0	1 2	1 1	0 0	1 4	3 0	0 3	2 8
N Metamyelocyte		30 2	13 4	11 6	12 6	18 6	3 6 8	15 2	21 8	29 6	14 4	33 0	3 6 3	39 0	29 8	29 1	25 6
E Metamyelocyte		1 6	1 6	0 9	0 0	0 0	1 0	1 2	1 2	1 2	0 4	0 6	0 0	0 8	0 2	0 6	1 8
PMN Neutrophil		8 0	3 6	4 7	6 0	2 2	8 2	11 8	5 4	1 6 0	2 8	9 9	9 5	9 6	14 2	3 5	7 0
PMN Lymphophil		0 2	0 6	0 2	0 0	0 0	0 4	0 8	0 0	0 6	0 4	1 1	4 7	0 4	0 2	0 0	0 2
Lymphocyte		4 8	2 6	6 9	40 8	3 0	6 4	10 0	2 8	10 0	5 6	3 5	9 0	9 8	7 2	12 7	2 0
Plasma cell		0 4	0 0	0 0	0 0	0 0	0 0	0 0	0 2	0 0	0 4	0 0	0 0	0 0	0 0	0 0	0 0
Monocyte		0 2	0 0	1 6	4 0	0 0	0 8	0 6	0 2	2 0	0 4	0 5	0 0	0 8	0 6	1 5	0 6
Reticulum cell		0 0	0 0	0 9	0 0	1 6	0 0	0 0	0 0	0 0	0 0	0 5	0 3	0 0	0 2	0 1	2 8
Mitotic cells		0 0	1 8	1 6	0 2	1 4	0 8	1 2	0 2	0 0	1 6	1 0	0 2	0 2	0 0	0 5	0 6
"Megakaryoblast"		0 0	22 6	12 4	0 0	20 2	4 2	3 2	0 4	0 0	11 2	5 7	1 5	0 0	0 0	9 0	1 5 2
Pronormoblast		0 4	2 2	2 7	0 2	4 2	5 2	0 8	0 4	0 0	1 6	0 6	1 3	0 2	1 0	0 8	0 8
B Normoblast		4 1	5 2	6 8	2 8	3 8	6 4	2 4	12 6	2 0	6 4	3 1	7 5	2 4	1 0	4 3	3 2
P Normoblast		38 0	30 8	42 3	25 6	19 6	17 2	44 8	44 2	35 4	45 2	27 0	13 0	25 0	32 6	20 9	23 4
O Normoblast		1 8	0 6	1 6	1 0	1 0	2 8	2 6	1 6	0 0	1 6	0 3	2 7	0 0	1 8	0 1	0 2
Leucocyte/Erythroid ratio		1 2	0 6	0 5	2 1	1 0	1 8	0 9	0 7	1 7	0 5	1 6	2 8	2 6	1 7	1 8	1 4

N Neutrophilic E eosinophilic PMN polymorphonuclear B basophilic P polychromatophilic O orthochromatic L E Liver extract  
 (15 units per cubic centimeter) in units PCA picrovioletamic acid in milligrams

rise in the volume of packed red cells to the level observed in pigs on the low protein diet and an increase in the leucocyte and neutrophil count occurred. There was no apparent increase in platelets. A secondary reticulocytosis was not elicited by the administration of 150 units of liver extract and no further increase in the volume of packed red cells was noted. A significant macrocytosis was not present in this animal at any time.

The effects of the various therapeutic agents on growth are shown in Fig. 5.

*Effect of Therapy on the Bone Marrow*—The effects of therapy on the bone marrow of each of the animals receiving the antagonist are presented in detail in Table VI.

The administration of liver extract was associated with a diminution in the number of megaloblasts in the marrow in each instance (Pigs 10 53, 10 54 and 10 56) but did not result in a complete disappearance of these cells. Cells were seen following liver therapy which had characteristics intermediate between basophilic normoblasts and megaloblasts. In two of the animals (10 53 and 10 54) the leucocyte erythroid ratio became more abnormal following liver therapy, whereas in the third (10 56) the ratio reverted to normal. In one pig (10 53) liver therapy had no apparent effect on the maturation of the leucocytes; in another (10 54) there was a significant increase in metamyelocytes followed later by an increase in neutrophils, whereas in the third there was a marked increase in metamyelocytes and a significant increase in neutrophils. In general the changes in the bone marrow following liver therapy were consistent with those seen in the peripheral blood.

Pteroylglutamic acid therapy resulted in a restoration of the bone marrow to normal in three instances. In one pig (10 53) although the megaloblasts disappeared completely and the leucocyte erythroid ratio returned to normal there was an increase in the percentage of lymphocytes and a decrease in metamyelocytes and neutrophils. In this animal the leucopenia and neutropenia in the peripheral blood persisted in spite of therapy.

At the termination of the experiment the animals were sacrificed and autopsies performed. There was no splenic enlargement and the marrow in the sternum, ribs, vertebrae and femurs was red and hyperplastic. There were no significant microscopic abnormalities in any of the organs examined which could not be attributed to a deficiency of protein.

#### DISCUSSION

The observations presented here indicate that there is a marked difference between the effect of pteroylglutamic acid and that of liver extract of the pernicious anemia type in pigs fed low protein diets and receiving a folic acid antagonist. The administration of liver extract was associated with a good reticulocyte response and a modest rise in volume of packed red cells in one animal (10 54) but in another (10 56) the effect was less impressive, and in a third (10 53) no significant change was observed in the blood even though a very large amount of liver extract, 150 units was given. The administration of pteroylglutamic acid on the other hand, was associated with a well pronounced hemopoietic response in these three animals as well as in another (10 64) not previously treated with liver extract.

These results are not in accord with the observations of Heinle, Welch, and co-workers<sup>7</sup> who concluded that "extrinsic factor and purified liver extracts are effective even though folic acid is not available to the animal." There are, however, several differences in the experimental conditions employed by the Cleveland group and by ourselves. Our animals received a diet low in protein. Presumably the three pigs reported by Welch, Heinle, and colleagues received adequate amounts of protein. Furthermore their animals were given Labco<sup>8</sup> 'vitamin-free' casein which contains little or no extrinsic factor,<sup>18</sup> while our animals were fed casein proved to contain substantial quantities of extrinsic factor. Finally, our animals were depleted of pteroylglutamic acid for a prolonged period prior to the administration of antagonist. Whether these differences in experimental procedure are significant or important cannot be stated at this time. It should be noted that the response of the one animal reported in detail by the Cleveland group was, for a pig, considerably delayed and gradual.<sup>6</sup>

From one point of view it may be considered surprising that any hemopoietic response to liver extract should have been observed in our animals. There is no reason to assume that there was in these animals a deficiency of intrinsic factor although this was not specifically investigated. They were fed what would seem to be, at least in terms of treating a patient with pernicious anemia, a rather substantial amount of extrinsic factor. One might ask whether under the conditions of the experiment, when a low protein diet, sulfasuxidine, and antagonist were administered, our animals actually absorbed an adequate amount of extrinsic factor. Since diarrhea was present absorption may not have been complete. The theoretic though remote possibility that extrinsic factor can be synthesized in the intestinal tract by bacteria must not be overlooked. If this were true, the administration of both sulfasuxidine and antagonist might seriously hinder such synthesis. In favor of the existence of a double deficiency that is both of extrinsic factor and of pteroylglutamic acid, is the fact that in the animal treated first with protein (10-53) there was no significant response to the subsequent administration of purified liver extract. However, contradicting this hypothesis is the fact that Pig 10-64 responded maximally to 20 mg of pteroylglutamic acid without prior feeding of increased amounts of casein. Furthermore, the administration of 150 units of liver extract sixteen days later failed to produce a secondary response. It may be mentioned here that in other experiments with pigs maintained on a low protein diet which were repeatedly depleted of mecin, we have observed no response to liver extract.<sup>19</sup>

A more plausible explanation of the activity of liver extract, such as it was, would be that a substance which it contains is concerned in some way with the utilization or availability of pteroylglutamic acid and that the administration of the large doses of liver extract made small tissue stores of pteroylglutamic acid available to the animal or that pteroylglutamic acid was released from more complex compounds. In patients with pernicious anemia in relapse Bethell and co-workers<sup>16</sup> as well as Welch and associates<sup>1, 2</sup> have observed that a substance in purified liver extract may contribute to the utilization of con

<sup>19</sup>The Borden Co. New York N. Y.

jugates of folic acid. The possibility that liver extract makes small amounts of pteroylglutamic acid available from the tissues is now under investigation in the current animal experiment.

Since the doses of liver extract used were large (75 to 150 units) it is unlikely that their unimpressive effect was due to inadequate dosage. The response which seemed to occur probably cannot be explained on the basis of the pteroylglutamic acid content of the liver extract since it has been shown that this preparation contains less than  $10 \mu\text{g}$  of microbiologically determinable *L. casei* factor per milliliter.<sup>8</sup> Furthermore if the response was due to pteroylglutamic acid or its precursors in the liver extract Pig 10 56 should have responded when 10 ml of liver extract were administered. Instead there was a slight initial response to 1 ml of liver extract and no further response when 10 ml were given eighteen days later. Again the complete lack of response to 150 units in Pig 10 53 is noteworthy.

It is of interest that the anemia observed was normocytic at first. Smears of the peripheral blood during this period revealed numerous large macrocytes and a comparable number of microcytes. After liver therapy in three animals definite macrocytosis appeared and this persisted beyond the period of reticulocytosis. It seems unlikely that the macrocytosis was due to liver therapy although this possibility has not been ruled out. A more plausible explanation would seem to be that sufficient time had not elapsed for a significant degree of macrocytosis to develop which would be manifest when mean values were determined. Provided the life span of the red cell of the pig is similar to that of man, that is about 120 days, replacement of the circulating normal sized red corpuscles by macrocytes with a corresponding increase in the mean corpuscular volume could not be expected in twenty to forty days. Since the liver had little or no effect on the anemia, a further period of forty six to sixty one days was available after liver therapy during which macrocytosis might develop. Although it is true that the one animal not treated initially with liver failed to develop macrocytosis it must be noted that this animal received the antagonist for only thirty five days prior to the administration of pteroylglutamic acid. It is of interest that the one pig reported in detail by Welch, Heinle and co workers<sup>9</sup> did not develop significant macrocytosis until the post treatment period.

The response of the leucocytes to liver extract and pteroylglutamic acid therapy is difficult to interpret. In several instances the values appeared to be rising at the time therapy was begun. In one animal there was no response to either substance. Although the anemia of the pig reported by Welch and associates<sup>9</sup> responded to the therapy given the leucocyte and neutrophil counts did not return to normal. It seems possible that a substance other than pteroylglutamic acid or the antipermeious anemia factor is involved. The platelet values in our animals are also exceedingly difficult to interpret. Their appearance to be a transient thrombocytosis in association with protein, liver or pteroylglutamic acid therapy followed by a decrease to an abnormally low level. More information is needed before definite conclusions can be drawn concerning the leucocytes and platelets.

There are various possible explanations of the relationship of liver extract factor and pteroylglutamic acid in hemopoiesis. It has been pointed out already, for example, that the liver factor may be concerned with the liberation of pteroylglutamic acid from conjugated forms of this substance in the tissue stores. If this were true, one would expect both liver extract and pteroylglutamic acid the substance it makes available, to be effective in producing a hemopoietic response in pernicious anemia. One would also expect that pteroylglutamic acid deficiency, if this included a deficiency of conjugates as well as of free forms of the vitamin, would not be affected by the administration of liver extract. If a deficiency of extrinsic factor were produced, one would expect a response to occur when extrinsic factor or liver extract or pteroylglutamic acid was given.

Such an hypothesis would be consistent with our own report<sup>20</sup> in which an anemic pig fed a highly purified diet responded to the administration of liver extract if we assume that that animal was deficient in extrinsic factor and was at most, only partially deficient in pteroylglutamic acid and its conjugates. It would explain the findings of Welch, Heinle, and co-workers<sup>6</sup> if it can be assumed that their animals were deficient in extrinsic factor and only partially deficient in pteroylglutamic acid and its conjugates, and it would be satisfied by our own observations, presented in this report, if it is assumed that our animals were mainly deficient in pteroylglutamic acid and its conjugates. The unimpressive effect of liver extracts and the variations in the different animals could be explained by assuming that only a moderate or slight deficiency of extrinsic factor was produced in our animals when a crude casein containing extrinsic factor was fed in low amounts.

Totter Sims, and Day,<sup>21</sup> on the basis of indirect evidence, have suggested that pteroylglutamic acid is concerned with the synthesis of protoporphyrin. The data on free erythrocyte protoporphyrin presented here do not substantiate this but admittedly do not disprove the theory.

It may be mentioned, in closing, that a pronounced ataxia appeared in one animal (10-64) approximately ten days after the administration of 20 mg of pteroylglutamic acid. A similar phenomenon has been observed in animals fed a diet deficient only in protein. This will be the subject of a separate report.

#### SUMMARY

Four pigs were maintained on a synthetic diet containing 10 per cent crude casein which was shown to possess extrinsic factor activity. The diet was supplemented with various vitamins, exclusive of pteroylglutamic acid, p-aminobenzoic acid, and inositol, sulfasuxidine and a pteroylglutamic acid antagonist were given as well. A severe anemia, leucopenia, and neutropenia developed. No such changes were observed in the blood of eight control animals maintained on the same diet plus pteroylglutamic acid, p-aminobenzoic acid, and inositol and to which no sulfasuxidine or pteroylglutamic acid antagonist was added.

Studies on the bone marrow of the animals receiving the pteroylglutamic acid antagonist revealed a marked reduction in the number of polymorphonuclear neutrophils and neutrophilic metamyelocytes, with a slight increase in the earlier forms of the myeloid series and a significant decrease in the leucocyte erythroid

ratio In addition, immature and nucleated red cells in many ways similar to those seen in the bone marrow of patients with pernicious anemia in relapse were observed

The anemia became more pronounced in spite of a great increase in the protein content of the diet of one animal It responded partially to the administration of liver extract in one animal questionably in another and not at all in a third Rapid relief of the anemia followed the administration of small doses of pteroylglutamic acid in all four pigs

Macrocytosis developed as the anemia progressed but at first the mean corpuscular volume was normal, probably because of the presence of many microcytes In the later stages of the disorder macrocytic anemia was demonstrated many more macrocytes than microcytes being present

The leucopenia and neutropenia were not permanently relieved by either protein or liver therapy In three of the animals the leucopenia and neutropenia were no longer present following the administration of pteroylglutamic acid In one animal the leucopenia and neutropenia persisted even after protein liver, and pteroylglutamic acid were given The platelets increased markedly after the administration of protein liver or pteroylglutamic acid but the increased levels were not sustained

The data suggest that the antipernicious anemia substance in purified liver extract does not completely if at all replace pteroylglutamic acid in the nutrition of the pig

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# A STUDY OF THE RESPONSE OF BACTERIAL POPULATIONS TO THE ACTION OF PENICILLIN A QUANTITATIVE DETERMINATION OF ITS EFFECT ON THE ORGANISMS

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THE action of penicillin on susceptible bacteria is still unknown despite the intense effort that is being made to discover the mechanism of its antibacterial action. While we fully realize the importance of a knowledge of the *modus operandi* of this valuable therapeutic agent we believe it equally important especially from the clinical standpoint to know what actually happens to the bacteria when exposed to the action of the drug. The first observation by Fleming<sup>1</sup> which led to the discovery of penicillin was that of lysis of staphylococci or destruction of organisms and yet the action of the antibiotic has been referred to since as bacteriostatic rather than bactericidal. The sensitivity tests on bacteria as performed today are evidently directed toward the determination of the inhibitory activity of the penicillin on the microorganisms. Even in assaying the antibiotics it is the inhibitory activity of the material on the standard test organism which is being considered. Results of penicillin sensitivity tests are reported in such terms as the minimum inhibiting concentration the titer of inhibition the minimum effective concentration and so forth. Some workers using a standard organism in their sensitivity tests compare the titer of the test with that of the standard and report their results as half as sensitive as the standard one quarter as sensitive and so on. Such reports only vaguely suggest the action of the drug on the bacteria. Although several accounts of the bactericidal effect of penicillin have recently been reported<sup>2</sup> the action is still referred to as bacteriostatic or interchangeably referred to as bacteriostatic and bactericidal.

It is the purpose of this communication to present experiments which show that it is possible to determine the exact condition of a bacterial population when exposed to the action of penicillin—the degree of sterilization the number killed and the number surviving the character of the survivors—and to discuss how these findings may influence and even lead to a modification of present day treatment of infectious diseases with the antibiotics.

## MATERIAL AND METHOD

The organisms used in the experiments were pneumococci from cases of lobar pneumonia hemolytic streptococci from scarlet fever and erysipela. *Streptococcus viridans* strain from cases of subacute bacterial endocarditis and staphylococci from general septicemias. All except one strain (*Str. viridans* c) were initial specimens that they were obtained from patients before penicillin treatment was instituted. The organisms were grown in phosphate buffered beef heart broth enriched when necessary with 3 per cent citrated horse plasma. Bacterial counts were made in nutrient agar poured plates, 3 per cent of citrated horse blood being added to the agar.

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Commercial sodium penicillin\* and streptomycin hydrochloride† were employed in the sensitivity tests, and penicillinase‡ was used to inactivate the penicillin in the culture.

Of the several methods now in use for determining the sensitivity of bacteria to the antibiotic, the serial dilution method is considered to be the most reliable since it allows a wide range of penicillin concentrations. This method, briefly, is as follows. Twofold serial dilutions of penicillin using broth as diluent, are made in small tubes. The number of tubes set up in a test depends on the range of penicillin concentrations desired, usually twelve. The penicillin dilutions are contained in 0.5 ml of broth. To each tube 0.5 ml of a young culture, a five to six hour growth, diluted to  $10^{-5}$  is added, giving a total of 1 ml in each tube. One tube containing culture without penicillin is added as control. The tubes are incubated at  $37^{\circ}\text{C}$  and are read the following day. The last clear tube in the series containing the least amount of penicillin which "inhibits" bacterial growth as judged by visual inspection is regarded as the indicator of the penicillin sensitivity of the organism tested. Two streaked plates are made from the last two clear tubes to "inure sterility."

Since visible lysis is a direct proof of destruction of bacteria, we decided first to subject each culture to such a test. For this purpose an opaque culture is needed to observe clearing. Diluting the culture, as is done in the described procedure, would not do, since the inoculated tubes look quite clear before incubation. Too many organisms (over night growth) on the other hand, would obscure lysis if it occurred only to a slight degree. A three to four hour culture, depending on the rate of growth of the particular organism, gave the desired turbidity and was used undiluted. The same culture was also tested when diluted to  $10^{-4}$  to compare the sensitivity titers. The tenfold serial dilutions were used in agar poured plates for bacterial counts. The number of organisms in the inoculum in different tests generally varied from 100,000,000 to 300,000,000 in the straight culture and from 10,000 to 30,000 in the diluted culture although smaller numbers were occasionally employed. The turbidity of the cultures was read before incubation and one tube was placed in the refrigerator to compare directly with the incubated tubes the following morning as a check on the turbidity reading. The tubes were incubated at  $37^{\circ}\text{C}$  for eighteen hours. The turbidity of the cultures was read by comparing each tube with a standard consisting of a set of fuller's earth suspensions in tubes ranging from a slight turbidity (25) to a density through which a dark object barely could be seen (500), twenty tubes in all. The titer of sensitivity, when straight culture was used for inoculation, was considered the smallest amount of penicillin which gave a turbidity reading equal to that of the initial culture before incubation.

Knowing that minute amounts of penicillin may inhibit growth of sensitive bacteria, we suspected that the inoculum used to subculture in normal media for sterility tests carried with it sufficient penicillin to inhibit growth and thus cause faulty interpretation of results. In order to eliminate this possibility we diluted the cultures exposed to penicillin so that the final concentration of the drug in the poured plates was reduced to negligible amounts. Penicillinase, used to inactivate the penicillin in the cultures, gave results similar to those obtained by dilution, and, since it was more convenient, this method was employed routinely for bacterial counts in 1 unit per milliliter amounts.

#### RESULTS OF THE EXPERIMENTS

As can be seen from Table I, the destruction of organisms by lysis occurred in all the bacterial strains tested except four, those of *St. viridans* strains identified as *salivarius*. Two greenish streptococcus strains one an enterococcus and the other unidentified, were lysed but in higher concentrations of penicillin. The extent of bacteriolysis varied in the different species, pneumococci showing the most, staphylococci less, and hemolytic streptococci least.

\*Wyeth Incorporated Philadelphia, Pa.

†Merck and Company, Inc. Rahway, N. J.

‡Schenley Laboratories, Inc. New York, N. Y.

TABLE I PENICILLIN SENSITIVITY TESTS SHOWING LAYS OF ORGANISMS, TURBIDITY READING AFTER 18 HOUR INCUBATION AT 37 C

ORGANISM	TURBIDITY BY FORF INCUBATION	PENICILLIN DILUTIONS BEGINNING WITH 60 UNITS/MI												INCUBATED CONTROL (NO PENICILLIN)	
		60	30	15	7.5	3.75	1.87	0.93	0.46	0.23	0.12	0.06	0.03		0.015
Str viridans a	50	50	50	50	50	50	50	50	50*	75	75	75	150	175	225
Str viridans b	50	50	50	50	50	50	50	50	50	75	75	100	125	150	200
Str viridans c	50	50	50	50	50	50	50	50	50	75	75	75	100	150	200
Str viridans d	50	50	50	50	50	50	50	50	50	75	100	125	150	175	175
Str viridans e	75	tr	tr	tr	tr	tr	50	125	125	150	500	500	500	500	400
Enterococcus	75	50	50	50	50	150	200	225	225	275	275	275	275	275	275
Staph aureus	75	25	25	25	25	25	25	25	25	25	25	25	75	275	300
Staph citreus	50	25	25	25	25	25	25	25	25	25	25	25	75	275	300
Hem strept ag	75	50	50	50	50	50	50	50	50	75	100	100	125	150	200
Hem strept li	100	75	75	75	75	75	75	75	75	75	75	100	125	150	225

ORGANISM	TURBIDITY BY FORF INCUBATION	PENICILLIN DILUTIONS BEGINNING WITH 20 UNITS/MI												INCUBATED CONTROL (NO PENICILLIN)	
		20	10	5	2.5	1.25	0.63	0.32	0.16	0.08	0.04	0.02	0.01		0.005
1 pneumo Type 1	100	25	25	25	25	25	25	25	25	25	25	25	50	50	225
1 pneumo Type 2	100	25	25	25	25	25	25	25	25	25	25	25	50	50	225
1 pneumo Type 3	100	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	50	50	225
1 pneumo Type 4	100	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	50	50	225
1 pneumo Type 5	100	25	25	25	25	25	25	25	25	25	25	25	50	50	225
1 pneumo Type 6	100	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	50	50	225
1 pneumo Type 7	100	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	50	50	225
1 pneumo Type 8	100	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	50	50	225
1 pneumo Type 9	100	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	50	50	225

tr Trace

tr Trace

Titer of macroscopic inhibition

Individual strains within the same species also differed but within a smaller margin. Lysis was not complete in any of the cultures, a residuum of viable cells always remaining even in the highest concentration of penicillin used that of 60 units per milliliter. In most instances the number of organisms surviving varied inversely with the degree of lysis.

Avery and Cullen<sup>8</sup> have shown that pneumococcal autolysin, while having no action on living pneumococci, has a powerful lytic action on heat-killed pneumococci. Bionfenbrenner and Muckenfuss<sup>9</sup> have shown the same to be true of staphylococci. It is, therefore, difficult to tell whether the lysis of these organisms was caused by the penicillin or whether the bacteria were first killed by the antibiotic and then autolyzed. It is possible that the lysis of pneumococci and staphylococci was a summation of effects of unfavorable influences on the bacterial cell. The lysis of hemolytic streptococci suggests that penicillin is capable of lysing bacteria by itself, since these organisms do not autolyze. Attempts to prepare autolysin from hemolytic streptococci have been unsuccessful.<sup>10</sup>

Table II represents a typical experiment performed with a *Str. viridans* strain. When the penicillin cultures were subcultured in normal media no growth appeared in the plates. Enough of the penicillin was evidently left in the tubes to still inhibit growth of the bacteria. However, when the penicillin in the tubes was inactivated with penicillinase and poured plates made, vast numbers of organisms were recovered, even from the tube containing 60 units per milliliter or 7500 times as much as the titer of "inhibition" (0.008 unit per milliliter).

When undiluted straight cultures used in the initial inoculum yielded a large number of residual organisms, a proportional number of viable bacteria were recovered from the penicillin cultures in which diluted inoculum was employed. In more sensitive strains, on the other hand, in those yielding smaller numbers of residual organisms no viable cells were found on subculture when a diluted inoculum was used. These findings indicate that individual organisms in a bacterial strain may differ in their reaction to the effect of the antibiotic. When cells of a certain type are present in small numbers they will be eliminated by high dilutions. Therefore the cells capable of withstanding large amounts of penicillin, being present in insufficient numbers to be carried through the serial dilutions, were excluded when the culture was diluted ten thousand times ( $10^{-4}$ ). See Tables II and III.

Higher titers of inhibition were obtained with diluted rather than with undiluted cultures. This did not, however, indicate the true extent of susceptibility of the bacterial strain tested. A comparison of Tables II and III demonstrates this clearly. Here the sensitivity of a hemolytic streptococcus may be compared with that of a *Str. viridans* strain. Both are susceptible to the same extent according to the dilution method. However, when the penicillin in the tubes was inactivated with penicillinase and bacterial counts were made, much greater numbers of viable organisms were recovered in the *Str. viridans* strain than in the hemolytic streptococcus strain—23 per cent

TABLE II REPRESENTATIVE TEST WITH A STR. VIRIDANS STRAIN (A) SHOWING NO GROWTH IN PENICILLIN CULTURES WITHOUT PENICILLINASE AND GROWTH WHEN THE DRUG WAS INACTIVATED WITH PENICILLINASE

CULTURES EXPOSED TO PENICILLIN (UNITS/ML)	CULTURAL PROCEDURE						TURBIDITY	
	NUMBER OF ORGANISMS INOCULATED						NUMBER OF ORGANISMS INOCULATED	
	30 000 000			3 000			10 000 000	3 000
	STREAKED PLATES	POURED PLATES WITHOUT PENICILLINASE	POURED PLATES WITH PENICILLINASE	STREAKED PLATES	POURED PLATES WITHOUT PENICILLINASE	POURED PLATES WITH PENICILLINASE	CULTURE UNDILUTED	CULTURE DILUTED TO 10 <sup>-4</sup>
60	-	-	7 000 000 colonies*	-	-	754 colonies	50	0
30	-	-	Not counted	-	-	816 colonies	50	0
15	-	-	Not counted	-	-	778 colonies	50	0
7.5	-	-	Not counted	-	-	902 colonies	50	0
3.75	-	-	Not counted	-	-	956 colonies	50	0
1.87	-	-	Not counted	-	-	938 colonies	50	0
0.93	-	-	Not counted	-	-	980 colonies	50	0
0.46	-	-	13 000 000 colonies	-	-	920 colonies	50	0
0.23	-	-	Not counted	-	-	Not counted	75	0
0.12	-	-	Not counted	-	-	Not counted	75	0
0.06	+	+	Not counted	-	-	Not counted	150	0
0.03	+	+	Not counted	-	-	Not counted	175	0
0.015	+	+	Not counted	-	-	880 colonies	200	0
0.008	+	+	Not counted	+	+	Not counted	225	175
0.004	+	+	Not counted	+	+	Not counted	50	0
No penicillin before incubation								
No penicillin after incubation							225	175

Bacterial counts subject to inherent error of method

- No growth + growth

Serial diluted for counting

TABLE III. REPRODUCIBILITY TEST WITH A HEMOLYTIC STRAIN (98) IN WHICH NO VIABLE ORGANISMS WERE FOUND WHEN INOCULUM WAS DILUTED TO  $10^{-10}$  BECAUSE OF SMALL YIELD OF RESIDUAL CELLS.

CULTURE EXPOSED TO PENICILLIN (UNITS/ML)	CULTURAL INCUBATION						TURBIDITY	
	NUMBER OF ORGANISMS INOCULATED						NUMBER OF ORGANISMS	
	100,000,000			10,000			300,000,000	100,000,000
	SPLIT PLATES	POURED PLATES WITHOUT INCHITINASE	POURED PLATES WITH INCHITINASE	SURFACED PLATES	POURED PLATES WITHOUT INCHITINASE	POURED PLATES WITH INCHITINASE	CULTURE UNDILUTED	CULTURE DILUTED TO $10^{-4}$
60	-	-	7,000 colonies*	-	-	-	50	0
30	-	-	Not counted	-	-	-	50	0
15	-	-	Not counted	-	-	-	50	0
7.5	-	-	Not counted	-	-	-	50	0
3.75	-	-	Not counted	-	-	-	50	0
1.87	-	-	Not counted	-	-	-	50	0
0.93	-	-	Not counted	-	-	-	50	0
0.46	-	-	Not counted	-	-	-	50	0
0.23	-	-	Not counted	-	-	-	50	0
0.12	-	-	140,000 colonies*	-	-	-	50	0
0.06	-	5 colonies	Not counted	-	-	-	75	0
0.03	-	-	Not counted	-	-	-	100	0
0.015	-	-	Not counted	-	-	-	125	0
0.008	-	-	Not counted	-	-	-	150	0
0.004	-	-	Not counted	-	-	-	175	0
No penicillin, before in- cubation	-	-	Not counted	-	-	15 colonies	200	200
No penicillin, after in- cubation	-	-	-	-	-	-	75	0
							200	200

- No growth

\* Serial diluted for counting.

survivals in the former as compared with a small fraction of 1 per cent in the latter. These facts may account for the general observation that bacteria tested by the present day methods show a greater sensitivity to penicillin *in vitro* than *in vivo*. The use of straight undiluted cultures in the tests gives results more approximating the sensitivity of bacteria *in vivo*.

The residual viable bacteria were found to be inhibited in low as well as in high concentrations of penicillin showing a wide range of inhibition. This was more evident in the tests in which the cultures used for inoculation were diluted. (See Table II.) It is probably due to the elimination of certain cells by the dilution, leaving a more uniform population.

Residual organisms (single colony culture) when retested that is on second exposure to penicillin showed no increase in resistance, or only a slight increase, and again yielded a residuum of viable cells similar to that of the original or parent strain. Lysis of cells, however, either did not occur, or occurred to a lesser degree than in the original culture. This again demonstrates the singular characteristic these residual cells have of being able to withstand the killing effect of the drug in high as well as in low concentration.

Since these findings point to the existence in a bacterial population of members differing in the manner in which they respond to the action of penicillin, it was thought advisable to test the residual bacteria which are capable of withstanding high concentrations of the drug with streptomycin in the hope that they might be susceptible to the latter antibiotic. For this purpose the following method was adopted.

Nutrient agar plates are seeded with an overnight growth of the culture to be tested by placing 0.3 ml. of the broth culture in the center of the plate and spreading it with a wire spreader so that an even, uniform film is produced. The plates are placed in the incubator for fifteen minutes with covers tilted for drying. Paper disks\* are impregnated with high concentrations of the drugs. A sterile pointed forceps is used in handling the disks. Each disk is immersed in the solution the excess being drained off against the wall of the tube. The disks are carefully placed on the surface of the inoculated plate four to a plate: two disks with penicillin solution 100 units per milliliter, one disk with streptomycin 100 units per milliliter and one disk with plain broth for control.

The following morning the zones of inhibition are measured and the paper disks used for residual organisms as follows. With sterile forceps each disk is carefully picked up and each placed into 10 ml. of sterile broth. The tubes are well shaken to free the bacteria which adhere to the paper disks. The original concentration of penicillin is thus diluted tenfold and by plating 1 ml. of the washing it is further diluted so that the final concentration in the plate is about one hundredth that of the original concentration in the disk. Not considering the amount of the drug which was absorbed by the agar. If the disk does contain some remaining penicillin it is in negligible amount so that the possibility of its acting on the subculture may be excluded.

\*Whatman filter paper No. 2 disk 10 mm. in diameter

TABLE IV  
EFFECT OF STREPTOMYCIN ON PENICILLIN RESIDUUM BACTERIA  
(PAIR DISKS\* IMMERSED WITH SOLUTION OF 100 UNITS/ML OF THE DRUGS)

BACTERIAL STRAIN	RESIDUUM CALLED BY DISK WITH DRUG	NUMBER OF ORGANISMS RECOVERED	ZONE OF INHIBITION	ORGANISMS CARRIED BY DISK WITHOUT DRUG (NORMAL CULTURE)	NUMBER OF ORGANISMS OBTAINED BY SERIAL DILUTION		
					10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>
<i>Str. viridans</i> a	Penicillin disk	14,000	32 mm	Normal culture	650	50	10
	Streptomycin disk	Immeasurable	None	Normal culture exposed to streptomycin, 10 units/ml	652	60	11
<i>Str. viridans</i> b	Penicillin disk	3,166	29 mm	Normal culture	556	72	8
	Streptomycin disk	Immeasurable	None	Normal culture exposed to streptomycin, 10 units/ml	592	62	2
<i>Str. viridans</i> c	Penicillin disk	1,363	28 mm	Normal culture	346	33	1
	Streptomycin disk	Immeasurable	None	Normal culture exposed to streptomycin, 10 units/ml	300	23	5
<i>Str. viridans</i> e	Penicillin disk	60,000	14 mm	Normal culture	188	18	0
	Streptomycin disk	Immeasurable	None	Normal culture exposed to streptomycin, 10 units/ml	188	15	3
<i>Enterococcus</i>	Penicillin disk	160	25 mm	Normal culture	151	43	1
	Streptomycin disk	Immeasurable	None	Normal culture exposed to streptomycin, 10 units/ml	166	10	5
<i>Str. viridans</i> f	Penicillin disk	820	30 mm				
	Streptomycin disk	Immeasurable	None				
Pneumo type 2	Penicillin disk	0	27 mm				
	Streptomycin disk	Immeasurable	None				
Pneumo type 10	Penicillin disk	20	12 mm				
	Streptomycin disk	Immeasurable	None				

\* Each pair of disks was immersed in 1 ml of the drug solution.

Five milliliters of each washing are used for plating 1 ml to a plate. One penicillin disk washing is plated with plain agar for residual cell count and another with agar containing streptomycin 10 units per milliliter, for its effect on penicillin residual organisms. The plates are then incubated overnight. The following morning they are examined, a colony count is made and the number found in the five plates is multiplied by two to get the total number of residual organisms from each paper disk. In order to obtain a ratio of the normal organisms originally in contact with the disk and the remaining viable cells after incubation with the penicillin, one paper disk moistened with broth is placed on a seeded plate and kept in the refrigerator for about one hour to allow the broth in the disk to be absorbed by the agar. The paper disk is then washed in the manner described; the washing is serially diluted and plated for bacterial counts. These are compared with the number of colonies obtained from the penicillin disks. The proportion of killed and surviving bacteria is thus obtained.

It is the belief of some investigators that antibiotics in the same concentration work more effectively on small rather than large numbers of organisms. For this reason we tested small numbers of the test bacteria with the same concentration of streptomycin as was used with the residual cells.

The results of the experiments are summarized in Table IV. While the normal cultures in small as well as in large numbers showed resistance to high concentrations of streptomycin, the residual bacteria were susceptible to low concentrations of the drug in amounts easily maintained in the body of the patient.

One of the six strains of *Str. viridans* tested did not respond to this treatment. The residual organisms of this strain were not affected by the streptomycin in the concentration used for the other cultures. To show that this method is reliable, two pneumococcus strains which by the tube method yielded small numbers of residual cells, were also subjected to this test. In one there were no viable cells found on the paper disk. The other yielded twenty colonies while the zones of inhibition were similar to those which yielded large numbers of viable residual organisms.

#### DISCUSSION

The results of the foregoing experiments bring to light several important facts in regard to the response of penicillin-sensitive organisms to the action of the drug.

We have shown that penicillin exerts a definite bactericidal effect on all susceptible strains in amounts possible to maintain in the body of the patient. It is capable of actually destroying the great majority of organisms by lysis, or otherwise under normal cultural conditions. However, there are always some cells left which are capable of withstanding the killing effect of even large doses of penicillin, remaining viable but unable to multiply in its presence. This inhibitory action extends through a wide range of concentrations, so that large and small amounts of the drug have the same inhibitory effect on these residual organisms.



The tests also indicate a heterogeneity of bacterial cultures in regard to the ability of individual members to react to penicillin. There seem to exist in most bacterial cultures cells of at least three types, differing from each other in the manner in which they react to the drug, namely (1) Those which are destroyed by lysis, (2) those which are killed without lysis, (3) those which are capable of withstanding the killing action of penicillin, remaining viable but unable to multiply in the presence of the drug. That this threefold response of penicillin sensitive bacteria is not a chance occurrence may be seen from the following findings. (1) When a bacterial culture, on exposure to penicillin, yielded a large number of residual viable organisms it always gave a proportional number of inhibited cells in the cultures for which small inoculum ( $10^{-4}$ ) was used. (2) In more sensitive strains, in those yielding small numbers of inhibited residual organisms, no viable cells were found in the cultures for which a small inoculum was used ( $10^{-4}$ ), indicating that, being present in small numbers in the original culture, these cells were eliminated by the dilution. See Table V.

TABLE V. SHOWING PROPORTIONAL NUMBERS OF SURVIVING ORGANISMS IN HIGH CONCENTRATIONS OF PENICILLIN WHEN LARGE AND SMALL INOCULUM WAS USED

BACTERIAL STRAIN	CULTURED IN PENICILLIN (UNITS/ML)	NUMBER OF ORGANISMS INOCULATED	NUMBER SURVIVED	RATIO OF SURVIVED TO KILLED
Str. viridans a	60	30,000,000	7,000,000	1/4
	60	3,000	724	1/4
Str. viridans b	60	66,000,000	6,000,000	1/11
	60	6,600	500	1/13
Str. viridans c	60	160,000,000	900,000	1/178
	60	16,000	60	1/268
Str. viridans d	60	164,000,000	600,000	1/273
	60	16,400	61	1/270
Staph. citreus	30	100,000,000	110,000	1/910
	30	10,000	9	1/1,100
Staph. aureus	60	95,000,000	7,000	1/13,500
	60	9,500	0	-
Enterococcus	60	11,200,000	6,400	1/1,750
	60	1,120	0	-
Hemolytic streptococcus 9S	60	300,000,000	7,000	1/43,000
	60	30,000	0	-
Pneumo Type 1	20	150,000,000	117	1/1,252,000
	20	15,000	0	-
Pneumo Type 3	20	105,000,000	128	1/820,000
	20	10,500	0	-
Pneumo Type 9	20	200,000,000	100	1/2,000,000
	20	20,000	0	-

- Eliminated by dilution

It was previously shown by one of us (S. S.)<sup>11</sup> that individual organisms in a bacterial culture, or specimen, also possess different degrees of sensitivity, or resistance, to sulfonamides. However, what makes penicillin a much more potent antibacterial agent is the fact that while the sulfa drugs are only bacteriostatic, penicillin actually destroys the sensitive organisms, leaving, in most cases, only a small number of viable cells capable of withstanding the action of the drug.

We believe that the success of penicillin therapy depends directly on the number of residual organisms left viable after the initial treatment with penicillin. The very small numbers of residual viable organisms in strains of pneumococcus, hemolytic streptococcus, and some strains of staphylococcus, as shown by our tests, may account for the dramatic results so often obtained with penicillin when used in the treatment of acute infections caused by these organisms. On the other hand, the very large numbers of viable organisms found in *Str. viridans* strains, as our tests indicate, may account for the protracted treatment with penicillin necessary in cases of subacute bacterial endocarditis in which *Str. viridans* is the most frequent etiological agent.

With the belief that individual organisms in a given culture possess different inherent characteristics in regard to drug susceptibility, we thought that the residual cells capable of withstanding large amounts of penicillin might perchance be sensitive to the action of other therapeutic agents. We therefore treated bacteria, which remained viable after exposure to penicillin with streptomycin. The results were striking. While the original parent strain was highly resistant to streptomycin, the residual penicillin inhibited organisms were destroyed by small amounts of streptomycin. The reason for this apparent discrepancy is probably the fact that the streptomycin sensitive cells were present in comparatively small numbers in the original culture so that the action of streptomycin on them was obscured by the overwhelming numbers of resistant cells. Six *Str. viridans* strains were subjected to this treatment and all but one gave similar results, as can be seen from Table IV. While only a small number of bacterial strains were thus tested we believe the results obtained so far warrant the recording of these findings. If the *in vivo* action of the antibiotics parallels the action *in vitro*—and the consensus of opinion is that it does—it is possible that these findings may have a practical application in the treatment of subacute bacterial endocarditis. The administration of large doses of penicillin, if the invader is penicillin sensitive, with interruption of the penicillin treatment once in its early stage by a short course of streptomycin therapy might possibly shorten the course of the disease and lead to permanent clearing of the infection. This would have to be preceded by proper sensitivity tests of both the original infecting organisms as well as the penicillin residual cells. A simple method for testing penicillin residual bacteria with streptomycin is included in this paper.

The present methods for testing the susceptibility of bacteria to the action of penicillin are inadequate since their results are not indicative of the true response of the organisms to the drug. By diluting the culture for inoculation the cells capable of surviving large amounts of penicillin being present in comparatively small numbers, are eliminated by dilution so that a true cross section of the bacterial population is not represented. Besides organisms capable of withstanding high concentrations of penicillin are also inhibited by low concentrations of the drug, so that streaking plates to insure sterility may carry over in the loop sufficient penicillin to inhibit growth on the plate, thus obscuring the actual condition of the cultures. By destroying the penicillin with

penicillinase or by diluting the cultures so that the amount of penicillin left in them is negligible, viable organisms may be recovered, bacterial counts made of the surviving cells, and the size of the residuum of viable bacteria determined. Only in this way may the true condition of a bacterial culture, when exposed to the drug, be determined.

In the method of comparing the titer of sensitivity of a test organism with that of a standard, a hemolytic streptococcus strain is usually employed. The fallacy of this method is discerned when the susceptibility of a hemolytic streptococcus is compared with that of a *St. viridans* strain (See Tables II and III). Both are sensitive to the same degree according to the dilution method of testing. However, when the numbers of residual viable organisms found in each are compared, they can hardly be called equally susceptible to penicillin—23 per cent survivals in *St. viridans* as compared with 0.05 per cent in the hemolytic streptococcus. The finding of a highly in vitro sensitive strain of *St. viridans*, as tested by the present methods, in a case of subacute bacterial endocarditis, which responds poorly to penicillin treatment, becomes less baffling when the true character of such a strain is observed.

#### SUMMARY

Experiments are presented which show that penicillin is capable of destroying susceptible bacteria, by lysis or otherwise, under normal cultural conditions in amounts possible to maintain in the body of the patient.

The destructive action of penicillin on sensitive bacterial strains, however, is not complete even in high concentrations of the drug. A residuum of viable organisms always remains which is capable of withstanding the destructive action of the antibiotic but is inhibited from multiplying in its presence.

This inhibitory activity of penicillin on the residual viable cells extends through a wide range of concentrations so that large and small amounts of the drug have the same inhibitory effect on these remaining organisms.

The findings suggest that bacterial cultures do not constitute a homogeneous population but that individual members may possess different characteristics as to the manner of reaction under the influence of the antibiotic.

The inferences to be drawn from the results of the experiments in relation to the treatment of infectious diseases with the antibiotics are discussed.

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## EFFECTS OF THE COLD PRESSOR TEST ON GLOMERULAR FILTRATION AND EFFECTIVE RENAL PLASMA FLOW

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THE cold pressor test is a well-known method of determining the ability of the vasomotor system to respond to reproducible stimuli.<sup>1,4</sup> Exposure of the hand or other body areas to ice water has been shown to cause pain, local vasoconstriction, and an elevation of arterial blood pressure.<sup>5,6</sup> Recently attention has been drawn to the effects of a local cold stimulus on renal function. Diminished urinary volume, increased specific gravity, decreased urea clearance values, and reduced minute chloride output have been observed following moderately prolonged exposure to the stimulus of the cold pressor test in pregnant and nonpregnant women. Equivocal observations of these phenomena have been reported on other subjects.<sup>8</sup> It is the purpose of this paper to report the effects of local peripheral cold on the specific renal functions of glomerular filtration and effective renal plasma flow.

### METHODS

The subjects for these experiments were healthy white male volunteers between the ages of 18 and 37 years who on physical examination and urinalysis showed no evidence of renal disease. The men were asked to abstain from all solid food and liquids, with the exception of one glass of water at bedtime, after supper on the evening prior to the experiment.

On the morning of the experiment the subject assumed a reclining position. An indwelling soft rubber catheter (5.5 mm in diameter) was installed in the bladder. Following the application of a blood pressure cuff to the left arm, intravenous infusions of isotonic saline were started in the veins of each forearm at a rate of 1 ml per minute. The needle in the left arm was used for drawing blood samples, that in the right, for the administration of test substances. While these procedures were being carried out (a period of about one hour), the subject ingested 1 liter of water. Then a priming dose of 40 ml of a 25 per cent mannitol solution\* and 3 ml of a 20 per cent sodium para-aminohippurate solution\* was administered intravenously within a period of five minutes. This was followed immediately by a sustaining infusion consisting of a mixture of 600 ml of isotonic saline, 100 ml of a 25 per cent solution of mannitol, and 16 ml of a 20 per cent solution of sodium para-aminohippurate at a rate of 4 ml per minute. This rate was maintained throughout the experiment.

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\*Obtained from Sharp & Dohme Inc Philadelphia Pa

Zero time was established at thirty minutes after the beginning of the priming dose. Six or seven consecutive clearance periods, of approximately fifteen minutes each, were carried out. After the first two or three of these periods, which served as controls, the subject's left foot was immersed to the level of the malleoli in stirred ice water at  $1^{\circ}\text{C}$  and was kept there throughout one entire period. Following removal of the foot from the cold stimulus, clearances were measured for three or four more periods.

Approximately five minutes after the beginning of each clearance period, blood samples were drawn through a three way stopcock attached to the needle in the left arm, care was taken to rinse out the system several times, using withdrawn and reinjected blood in order to wash out any residual saline. Time was noted, to the nearest tenth of a minute, at the beginning and end of the drawing of each sample and the average was taken as the blood sampling time. At the end of each period the bladder was washed with 20 ml of saline and 20 ml of an

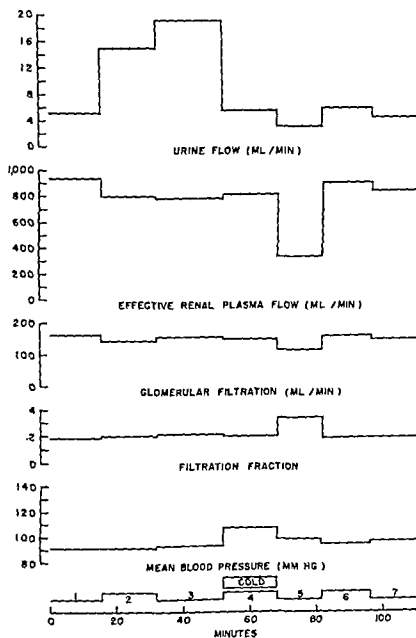


Fig 1—Representative experiment

Auscultatory blood pressures were obtained at least twice during each of the control periods as well as within thirty seconds before and after immersion of the foot in the ice water. During the period of immersion and in the two

TABLE I SUMMARY OF EXPERIMENTS

SUBJECT	DATE	CONDITION*	NUMBER OF PERIODS	AVERAGE DURATION (MIN)	MANITOL		P.A.H.		FILTRATION FRAC. (TON†)	AVERAGE BP ‡ (MM Hg)	CHANGE OF $C_{50}$ (%)	CHANGE OF $C_{50}$ (%)	CHANGE OF AV BP (%)
					PLASMA (MC / ML)	CLEARANCE (ML / MIN)	PLASMA (MC / ML)	CLEARANCE (ML / MIN)					
1	9/22/47	a	3	16.6	635	164	0360	711	23	101	-9	-24	+8
	9/22/47	c	2	15.3	671	150	0323	512	28	109	+15	+22	+2
	9/22/47	p	2	14.9	687	189	0252	864	22	104			
2	9/23/47	a	3	16.8	725	152	0280	922	19	95	-12	-32	+13
	9/23/47	c	2	14.9	801	133	0292	562	27	107	-3	+3	0
	9/23/47	p	2	15.5	821	117	0251	843	19	95			
3	9/24/47	a	2	15.9	463	189	0233	637	30	116	+2	+4	+15
	9/24/47	c	3	14.5	405	192	0190	663	29	133	-13	+4	+4
	9/24/47	p	2	15.1	641	164	0188	663	25	120			
4	9/25/47	a	2	15.0	513	182	0165	696	26	104	-28	-27	+13
	9/25/47	c	2	15.0	519	132	0141	309	26	117	-15	-7	+2
	9/25/47	p	2	14.8	546	154	0178	645	24	106			
5	9/29/47	a	2	15.0	744	153	0193	926	17	106	-23	-23	+17
	9/29/47	c	1	15.3	770	119	0180	711	17	124	-23	-23	+17
	9/29/47	p	2	14.8	936	117	0192	737	16	124	-16	-7	+8
6	9/30/47	a	2	14.8	972	128	0170	862	15	115			
	9/30/47	c	2	15.1	502	179	0150	991	20	90	-14	-26	+20
	9/30/47	p	2	15.3	590	154	0175	566	27	108	+7	+7	-1
7	10/6/47	a	2	15.9	680	191	0160	790	25	80			
	10/6/47	c	3	15.2	791	116	0244	627	19	95	-12	-22	+12
	10/6/47	p	2	16.0	810	102	0236	486	21	106	-2	+5	+3
Average % of control		c			971	114	0266	655	17	98	-14	-21	+14
Average % of control		p									-1	+4	+3

\*a Control periods c periods of cold effect p recovery periods

† Filtration fraction =  $\frac{\text{Manitol clearance}}{\text{I.V.H. clearance}}$ 

‡ (Systolic + diastolic) / 2 = Mean BP as rate of all readings

§ Change from control (a) by 10%

|| Change from control (a) by 5%

¶ Change from control (a) by 10%

||| Change from control (a) by 5%

|||| Change from control (a) by 10%

following periods determinations were made at approximately two minute intervals. Thereafter the frequency of the readings was similar to that during the control periods.

Analyses for mannitol and sodium para aminohippurate were carried out on heparinized plasma and on diluted urine samples according to the methods of Corcoran and Page<sup>9</sup> and Smith and co workers<sup>10</sup> respectively. It has been suggested that the clearance of mannitol may be slightly lower than the true glomerular filtration rate.<sup>11</sup> This would not alter the interpretation of these experiments since the importance of these data lies in their relative rather than in their absolute values.

### RESULTS

The results of these experiments are summarized in Table I. A representative experiment is shown in Fig. 1. As will be noted in six out of seven subjects both glomerular filtration rate and effective renal plasma flow decreased either during the application of the cold stimulus or within approximately thirty minutes thereafter. In no subject did the effect persist longer. The average decreases in glomerular filtration rate and effective renal plasma flow as compared with the controls were 14 per cent and 21 per cent respectively. The observed depression of urine flow confirms the finding of Odell and Aragon.

In all subjects the blood pressure rose promptly after application of the cold stimulus and this rise was sustained throughout the period of immersion. Upon removal of the stimulus the blood pressure gradually decreased returning to control levels in fifteen to twenty minutes. Examination of these data reveals no correlation between the degree of blood pressure elevation and the observed changes in renal function.

### DISCUSSION

The application of a peripheral cold stimulus is found to decrease urinary minute volume, glomerular filtration rate and effective renal plasma flow.

The large reduction of urine flow as compared with the moderate depression of glomerular filtration rate is regarded as evidence of alteration in the tubular reabsorption of water. This antidiuretic response may be of the same nature as that demonstrated by Rydin and Verney in dogs subjected to emotional stress.<sup>12</sup>

The results obtained on Subject 3 (26 years of age) may be of interest. While the blood pressure rose in all experiments as a result of the stimulus the rise was not associated with changes in glomerular filtration and renal plasma flow in this one subject. The initial diastolic blood pressure (128/90) together with the blood pressure response (164/116) during exposure to cold would suggest according to the criteria of Hines and Brown<sup>13</sup> that this individual belongs to the prehypertensive group. However as indicated above these observations do not establish a correlation between the degree of blood pressure rise and the changes in renal function in response to the cold stimulus.

The mechanisms ultimately responsible for these findings remain to be identified and are being investigated.



## SUMMARY

Studies were made of the effects of the cold pressor test on renal function. Seven male volunteers who had no history of renal disease served as subjects. Glomerular filtration (as measured by mannitol clearance) and effective renal plasma flow (as measured by sodium para-aminohippurate clearance) were determined before, during, and after immersion of the foot in ice water at 1° C for fifteen minutes.

In six out of seven subjects both glomerular filtration rate and effective renal plasma flow decreased either during the application of the cold stimulus or within approximately thirty minutes thereafter. In no subject did the effect persist longer. The average decreases in glomerular filtration rate and effective renal plasma flow, as compared with the control values, were 14 per cent and 21 per cent, respectively.

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## RELATION BETWEEN STRUCTURAL AND FUNCTIONAL ALTERATIONS OF THE LIVER

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A CORRELATION between basic histopathologic phenomena in the liver and the results of the different liver function tests is still not established. In the past, repeated attempts to secure such a correlation were primarily based upon animal investigations with experimentally produced well defined conditions.<sup>1-4</sup> In the human being, several approaches were attempted. One approach was the comparison between the histologic picture found in autopsy material and the results of liver function tests carried out shortly before death. This is objectionable because of the marked changes which occur during the agonal period.<sup>5</sup> Another approach was the performance of serial function tests during the course of a disease with well established histologic pictures as for example infectious hepatitis or obstructive jaundice.<sup>6-9</sup> Only recently has a systematic attempt been made to compare the results of function tests with the histologic picture seen in biopsies.<sup>10-12</sup> In the following study, utilizing a relatively large series of cases of different diseases a statistical attempt has been made to compare morphologic phenomena, independent of the underlying disease with the results of liver function tests carried out at the time of the biopsy. The utilization of a larger material appeared desirable to overcome the obvious overlapping caused by the occurrence of more than one of the basic histologic phenomenon in a given case.

A statistical correlation between morphologic alterations and the results of liver function tests was carried out to study the following two problems

- 1 Evaluation of different liver function tests by comparison of their results with the presence and degree of liver cell damage
- 2 Functional significance of the different basic morphologic phenomena

### MATERIAL AND METHODS

Patients suffering from various stages of different liver diseases or hepatomegalies make up the material of this study. The liver diseases studied include acute infectious and toxic hepatitis, extrahepatic biliary obstruction due to tumor or stone, different types of cirrhosis and a miscellaneous group which included such conditions as amyloidosis, Boeck's sarcoid and lymphosarcoma xanthomatosis and so on. A total of one hundred sixty five biopsies which included thirty five repeat biopsies on the same patients was performed and the histologic findings were statistically correlated with the results of a series of function tests carried out within two days of the biopsy. All function tests were not carried out in every case. The biopsies in the great majority of cases were performed by means of the Turkel needle<sup>13</sup> utilizing the lateral approach through the seventh to tenth right costal inter space. In a few

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instances they were performed during laparotomy. The biopsy material was fixed in Zenker formalin or Carnoy's solution. Sections were stained with hematoxylin-eosin, Mallory's aniline blue connective tissue stain, and Gomori's reticulum fiber stain. The following histopathologic phenomena were correlated with results of liver function tests: (1) diffuse liver cell damage, (2) focal necrosis, (3) regeneration, (4) distorted reconstruction, (5) perportal inflammatory activity, (6) fatty metamorphosis, and (7) increased Kupffer cell activity.

A tetrachoric coefficient<sup>14, 15</sup> of correlation of these phenomena with results of the biochemical determination was chosen for statistical correlation. By this method one is enabled to correlate the presence or absence of one variable (histopathologic phenomena) with the presence or absence of a second variable (abnormal result of function test). The coefficient, when corrected for the number of cases, yielded a critical ratio (CR). This mathematical figure (CR) indicates the degree to which the obtained results represent a reliable or consistent trend. A critical ratio above 2 is considered significant. The formula employed and an example in the derivation of a CR value follows:

$$CR = \frac{ad - bc}{\sqrt{(a+b)(c+d)(a+c)(b+d)}} - \frac{1}{\sqrt{N}}$$

Variable 2	Variable 1				Focal Neurons	Sedimentation Rate						
	+	-	b	a + b		Present	Abnormal	Normal	Total			
							c	d	c + d	77	57	134
	a + c	b + d	N									

$$CR = \frac{1060 - 888}{\sqrt{16,984,440}} - \frac{1}{\sqrt{134}} = +0.482$$

For use in scatter graphs the pathologic phenomena were subjectively graded from 1 plus to 4 plus.

Limits between normal and pathologic results of each of the applied function tests were arbitrarily drawn (Table I). In general, the borderline of the pathologic levels chosen is the one currently accepted. In some instances, however, the borderline has been set higher than that usually employed since only the markedly pathologic levels were expected to show a correlation. This was especially true in the case of total serum bilirubin and also cholesterol-ester ratio, prothrombin time, and sedimentation rate. For serum alkaline phosphatase two levels were selected: 4 to 10 Bodansky units as found as a rule in liver cell damage, and

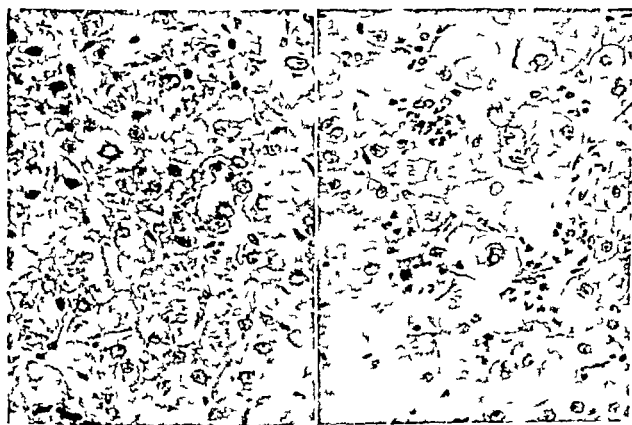
TABLE I FUNCTION TESTS AND LEVELS ARBITRARILY SELECTED FOR CORRELATION WITH HISTOPATHOLOGIC PHENOMENA

Serum total protein	Below 6 Gm %
Albumin/globulin ratio	Below 1.0
Serum nonprotein nitrogen	Above 35 mg %
Cephalin-cholesterol flocculation	+++ and ++++ <sup>16</sup>
Thymol turbidity	Above 4 units <sup>17, 18</sup>
Total serum cholesterol	Above 250 mg %
Percentage of cholesterol ester in total serum cholesterol	Below 45% <sup>19, 20</sup>
Serum alkaline phosphatase	Above 10 BU <sup>21</sup>
Total serum bilirubin	Above 8 mg % <sup>22</sup>
Bromsulfalein retention (5 mg/kg in 45 min)	Above 10%
Urinary urobilinogen	Above 3 Ehrlich units <sup>23</sup>
Stool urobilinogen	Below 10 Ehrlich units <sup>23</sup>
Prothrombin time	Below 80% <sup>24</sup>
Serum vitamin A	Below 15 µg <sup>25</sup>
Sedimentation rate	Above 30 mm/hr

above 10 unit as found usually in extrahepatic biliary obstruction. Decreased pathologic values of urinary urobilinogen were not utilized in this study. However a reduction of stool urobilinogen to below 10 units was considered evidence of biliary obstruction and was used in the statistical correlation. The level selected for serum vitamin A was quite low. Abnormal elevation of vitamin A was not found in this series.

#### RESULTS

*Diffuse liver cell damage* (Fig 1 A) was considered to be present when all or almost all liver cells revealed one or more of the following aberrations from the normal: dissociation of the liver cell cords; unusual variation in size of the cells; hazy borders; irregular staining of the cytoplasm with appearance of irregularly shaped vacuoles (apparently not containing fat); clumping of cytoplasm with marked basophilia of the clumped material; eosinophilic coagulation necrosis; irregular size, shape and staining of the nuclei leading to pyknosis.



A

B

Fig 1—Photomicrographs of liver biopsy specimens. A: Diffuse liver cell damage in a case of infectious hepatitis. Cytoplasm and nuclei of the liver cells vary in size and staining qualities. The outline of the cells is hazy and the architecture of the cord is irregular. B: Focal necrosis in a case of cholecystitis. The liver cells have a normal appearance. In small areas they are necrotic and are replaced by polymorphonuclear leucocytes which accumulate in the perisinusoidal spaces.

or ballooning of the nuclei (glycogen nuclei) and hyalinization of the entire cell. These changes varied in intensity throughout the lobule but some degree of damage was present in the entire lobule.

As seen from Table II there was a statistically significant correlation between liver cell damage and cephalin-cholesterol flocculation, thymol turbidity, albumin/globulin ratio and bromsulfalein retention. A less significant relation was found to marked decrease in prothrombin, high values of serum bilirubin and slightly elevated levels of alkaline phosphatase (4 to 10 Bodansky units). Levels of alkaline phosphatase above 10 units showed little correlation

TABLE II CORRELATION BETWEEN MORPHOLOGIC PHENOMENA AND PATHOLOGIC RESULTS OF LIVER FUNCTION TESTS AS EXPRESSED BY THE CRITICAL RATIO (A RATIO ABOVE +2 INDICATES A SIGNIFICANT RELATION)

	LIVER CELL DAMAGE	FOCAL NECRO SIS	REGEN ERATION	DIS TOPTED RECON STRUCT TION	PORTAL ACTIVITY	FATTY CHANGES	KUPFER CELL ACTIVITY
Total protein	-2.64	-3.07	-1.99	-0.82	-0.43	+0.56	-0.20
A/G ratio	+4.19	+0.43	+0.28	+0.81	+0.78	-1.65	+4.57
Nonprotein nitrogen	+1.23	-1.91	+0.61	-1.32	-2.54	-1.40	-0.45
Cephalin cholesterol flocculation	+4.39	+1.53	-0.56	+4.47	+1.92	+1.68	-0.50
Thymol turbidity	+5.15	+0.14	+2.87	+2.97	+0.31	+1.27	+1.97
Total cholesterol	-1.03	+1.36	+1.36	-2.81	-2.15	+0.52	+1.45
Cholesterol esters	+0.59	-2.39	+0.21	+0.50	+1.27	+0.80	+0.01
Alkaline phosphatase (as a whole)	+0.27	-0.27	+0.60	+0.75	-3.17	-0.96	-0.19
Alkaline phosphatase (4-10 BU)	+2.54						
Alkaline phosphatase (above 10 BU)	+0.05						
Serum bilirubin	+2.52	-2.36	+1.16	-1.67	+0.38	-2.41	+3.54
Bromsulphalein	+3.92	-0.90	+0.52	-0.87	+0.50	-0.19	+0.03
Urinariy urobilinogen	-0.98	-1.42	-2.65	+1.83	-0.85	-0.97	-0.11
Fecal urobilinogen	-0.12	-2.48	+1.41	-1.17	-1.71	+0.12	-0.44
Prothrombin time	+2.09	-2.23	-1.88	+0.62	+1.42	-0.16	-0.03
Plasma vitamin A	+2.67	-0.36	+1.42	+1.14	+0.67	-0.28	+0.53
Sedimentation rate	+0.02	+0.48	+0.16	+2.77	+2.95	-0.51	-4.63

as did the results of the alkaline phosphatase when taken as a whole. There was no relation between histologically recognizable parenchymal damage and abnormal levels of total protein, urinary urobilinogen, decrease in cholesterol ester ratio, elevated nonprotein nitrogen, and sedimentation rate.

Among the scatter graphs which plot the quantitative relation between liver cell damage and the results of various liver function tests, three appear particularly instructive. No quantitative relation was found between parenchymal damage and total serum protein levels (Fig. 2), except at very low values (cases of hypoproteinemia due to causes other than liver disease were not included in this study). The degree of morphologic damage associated with total protein levels between 5 and 8 per cent was similar. However, there was an inverse quantitative relation between the albumin/globulin ratio and the degree of parenchymal damage (Fig. 3). The relation between liver cell damage and cephalin cholesterol flocculation is more complicated (Fig. 4). The plotted (composite) curve shows that the degree of liver cell damage is proportional to the cephalin-cholesterol flocculation between the ranges of 1 plus and 4 plus. However, a greater degree of damage coincided with cephalin-cholesterol flocculation values of 0 than those of 1 plus. The explanation for this may lie in the fact that many patients with negative cephalin-cholesterol flocculation had extra hepatic biliary obstruction secondary to tumor or lithiasis and some of these morphologically exhibited marked liver cell damage. On the other hand, most of the cases in the 1 plus to 4 plus range had primary hepatitis or cirrhosis.

*Focal necrosis* (Fig. 1, B) was indicated by small, irregularly scattered areas in which the parenchymal liver cells were either absent or present in the form of small anuclear fragments. They were replaced by large numbers of round

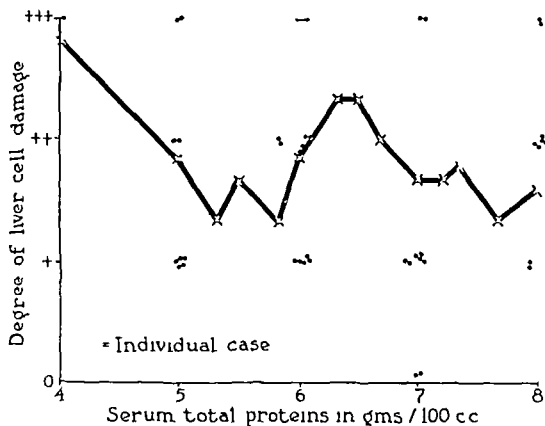


Fig 2—Relation between serum total proteins and liver cell damage

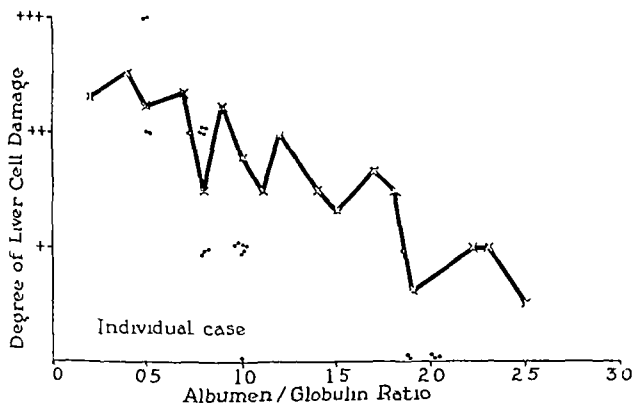


Fig 3—Relation between albumin/globulin ratio and liver cell damage

cells or polymorphonuclear leucocytes giving these areas a rather cellular appearance. No definite correlation between focal necrosis and any of the function tests was found.

*Regeneration of liver cells* (Fig 5, A) was characterized by large parenchymal cells often containing more than two nuclei which were frequently bizarrely shaped. These regenerating cells were found either in protracted liver cell damage or in apparent recovery from a preceding injury. This morphologic phenomenon showed a correlation only with elevated levels of the thymol turbidity test.

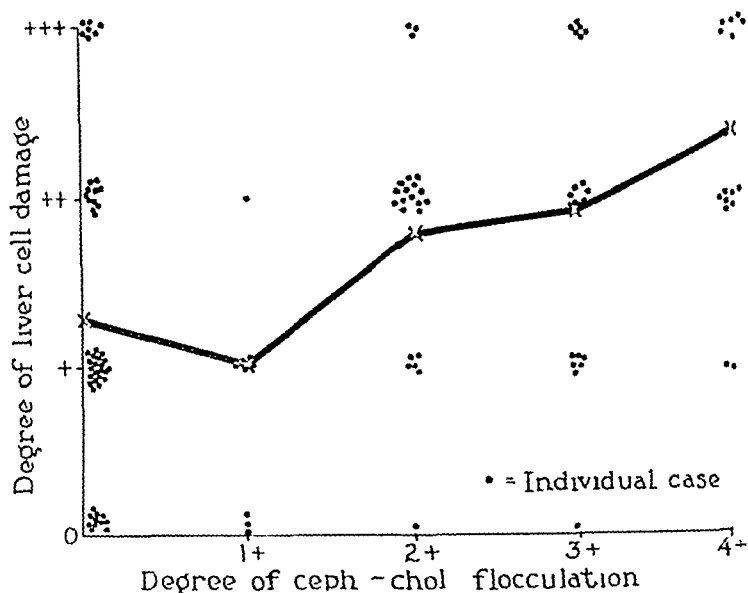


Fig 4—Relation between cephalin cholesterol flocculation and liver cell damage

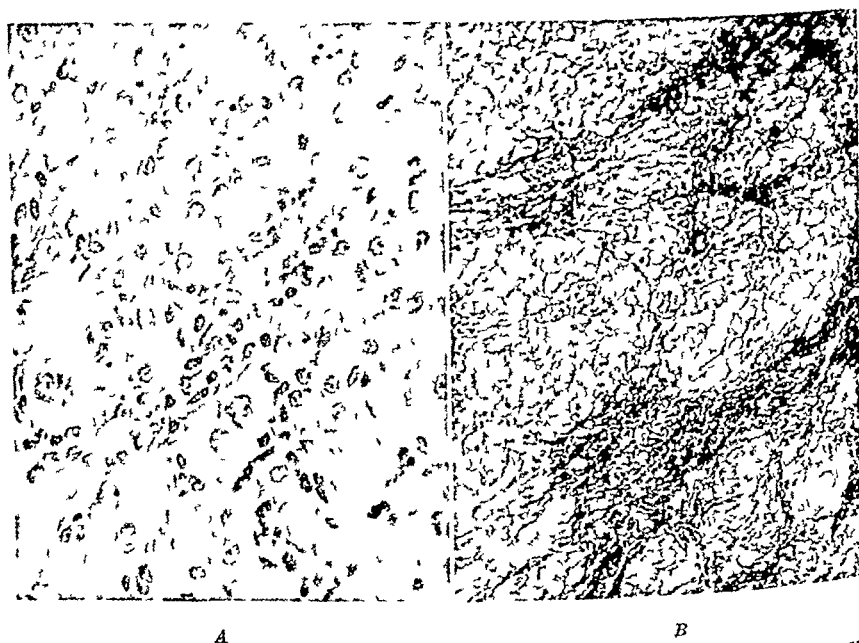


Fig 5—Photomicrographs of liver biopsy specimens. A Marked liver cell regeneration in a case of chronic infectious hepatitis. The liver cells in general reveal little damage but some of them are large and have two or three nuclei and abundant cytoplasm. There is some proliferation of histiocytic cells. B Distorted reconstruction in a case of portal cirrhosis (Mallory's aniline blue stain). Small nodules devoid of the usual lobular architecture are separated by wide connective tissue trabeculae.

*Distorted reconstruction of the liver parenchyma* (Fig 5 B) denoted partial or complete loss of the lobular pattern with replacement by parenchymal nodules, the cells of which were not arranged around a central vein. These pseudolobules varied in size, were often found near the periportal field and were usually well demarcated by more or less dense connective tissue trabeculae.

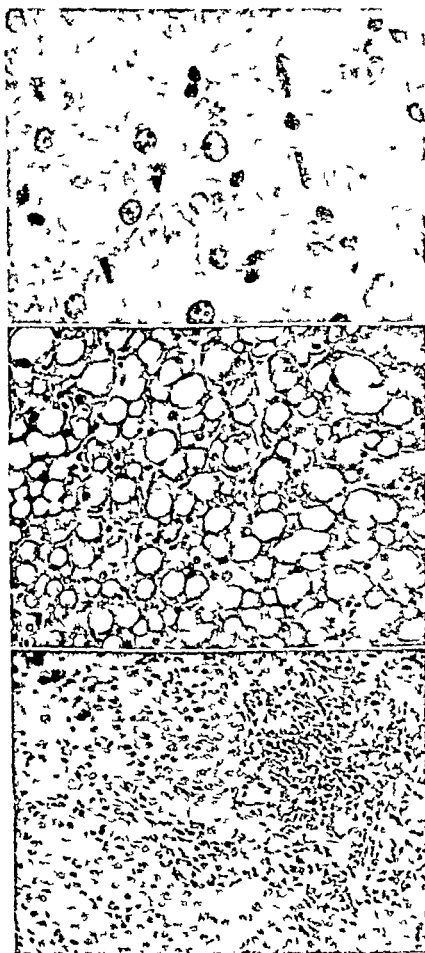


Fig. 6.—Photomicrographs of liver biopsy specimens. A: Periportal inflammatory activity. There is dense lymphocytic and histiocytic infiltration of the widened periportal field. The inflammatory exudate is arranged around lymphatics. There is some proliferation of the septal bile ducts. B: Fatty metamorphosis in a case of acute toxic hepatitis. Large fat droplets replace the cytoplasm of most of the liver cells. C: Kupffer cell mobilization (high power). The liver cells reveal normal appearance. The Kupffer cells are separated from the liver cell cords. They are large and bulge forward into the sinusoids.



This phenomenon occurred in cirrhosis. Distorted reconstruction revealed significant correlation with cephalin-cholesterol flocculation and a less significant one with thymol turbidity and sedimentation rate.

*Periportal inflammatory reaction* (Fig 6, A) described inflammatory changes in the portal triads usually originating in and around the lymphatics. These changes were characterized by accumulations of round cells and only rarely of polymorphonuclear leucocytes. The cellular accumulations varied in size and shape. Proliferation of septal bile ducts was frequently associated with the more marked of these inflammatory changes. The reaction showed a significant relation to increased sedimentation rate and only a minor one to the cephalin-cholesterol flocculation test.

*Fatty metamorphosis* (Fig 6, B) was characterized by accumulation of fat in the liver cells in the form of various sized droplets. There appeared to be no significant relation between these fatty changes and any of the function tests.

*Increased Kupffer cell activity* (Fig 6, C) denoted their mobilization and proliferation. This was characterized by an increase in the number and an enlargement of the individual Kupffer cells. They were separated from the liver cell cords and their abundant cytoplasm extended into the lumen of the sinus and often contained phagocytosed material. This picture showed a significant correlation with the albumin/globulin ratio and with a marked increase in serum bilirubin.

#### COMMENT

In the interpretation of the correlations found, it must be kept in mind that they are statistical and association must not be confused with causation. The fact that a morphologic phenomenon may be associated with abnormal values of a certain function test is no proof that the abnormal function is caused by that pathologic change. Some common factor might be responsible for both morphologic change and functional alteration.

As expected, diffuse liver cell damage revealed, in general, a good correlation with many of the liver function tests. In some instances, however, liver function tests were negative in the presence of visible liver cell damage. Moreover, in individual cases there were various combinations of positive liver function tests. The pattern of these variations is only partly known. Thus the cephalin cholesterol flocculation test was almost invariably negative in uncomplicated extrahepatic biliary obstruction.

Focal changes, though morphologically often far more impressive than generalized liver cell damage (the recognition of which is not always a simple matter), are not necessarily associated with liver function impairment. The bulk of the still intact liver parenchyma compensates for the relatively few cells which are destroyed. This observation is well in keeping with the widespread occurrence of focal necrosis in a multitude of diseases in which no functional liver impairment is found. Hence, conclusions concerning the extent of liver cell damage should not be drawn from isolated though anatomically impressive alterations.

Fatty changes as such do not interfere markedly with liver function. Distorted reconstruction as an expression of cirrhosis is related to some but not to all of the function tests. Except for the thymol turbidity test there is no statistically recognizable relation between regenerative processes and any of the function tests. The increased Kupffer cell activity in cases of severe jaundice is probably best explained by the large amounts of bile pigment in them. The inflammatory nature of the portal reaction explains its correlation with the elevation of the sedimentation rate, however, no relation to other function tests was demonstrated.

The evaluation of the various function tests by a morphologic method rendered, in general, results similar to those of other methods.<sup>6, 28</sup> In contrast, however, to the findings of Sherlock<sup>10</sup> this method failed to reveal a significant correlation between total serum protein concentration and any of the examined pathologic phenomena. The albumin/globulin ratio appeared to be a much better index of liver cell damage. The total protein concentration without albumin/globulin partition is no indicator of liver cell damage except when markedly decreased. With marked liver cell damage the albumin/globulin ratio may be reversed with normal total protein values. This is partly explained by reduced formation of albumin by the damaged liver. The demonstrated correlation between Kupffer cell mobilization and the albumin/globulin ratio is probably due to hyperglobulinemia which is known to occur with stimulation of the reticuloendothelial system.<sup>29</sup> The nonprotein nitrogen appears to be unrelated to any of the examined phenomena. Elevation of nonprotein nitrogen and urea nitrogen in liver disease (in some forms of hepatitis and in prolonged extrahepatic biliary obstruction) is primarily a renal phenomenon due to pathologic reabsorption of urea in the renal tubules.<sup>30, 31</sup>

The cephalin cholesterol flocculation revealed a good correlation with liver cell damage since in the absence of parenchymal damage the cephalin flocculation is almost invariably negative. This observation obtained by statistical evaluation of the entire material must be qualified when individual cases are analyzed. The cephalin cholesterol flocculation is usually negative in uncomplicated extrahepatic biliary obstruction, even if severe liver damage is visible under the microscope. With equal degrees of morphologically demonstrable liver damage the cephalin cholesterol flocculation is pathologic in primary hepatitis (infectious or toxic) or in cirrhosis and normal in an uncomplicated biliary obstruction due to tumor or stone. If the biliary obstruction is complicated by bacterial infection of the portal triads the cephalin cholesterol flocculation becomes positive.<sup>12</sup> The aforementioned characteristics of the cephalin-cholesterol flocculation test make it especially useful from a practical diagnostic standpoint.<sup>3, 3</sup> No explanation can be offered for the fact that a test which depends on the relation between albumin and gamma globulin was negative in prolonged uncomplicated biliary obstruction despite advanced liver cell damage. The fact that this test is usually positive in cirrhosis accounts for its close correlation with distorted reconstruction of the lobular pattern.

The thymol turbidity reveals statistically the most significant correlation with liver cell damage. The close correlation with regeneration agrees with

observations that this test remains positive in infectious hepatitis longer than the other tests. Based on this, Kunkel and Hoagland<sup>36</sup> associated the thymol turbidity with regeneration. Necfe,<sup>37</sup> however, connected it with periportal infiltration, with which we found no correlation. Whether the thymol turbidity is an expression of regeneration also in the early stages of infectious hepatitis is as yet unsettled. Its relation to reconstruction can be interpreted similarly to that of the cephalin-cholesterol flocculation test.

The total serum cholesterol and cholesterol ester fraction showed no significant correlation with any of the examined morphologic alterations. The total cholesterol elevation in surgical jaundice is probably due to obstructed bile flow. Cholesterol ester reduction in the material studied appeared too erratic to be statistically significant.

Elevation of serum alkaline phosphatase in general showed no relation to the studied morphologic changes. However, if the results of the alkaline phosphatase tests are broken down, the group between 4 and 10 Bodansky units revealed a relation to liver cell damage but not the group above 10 Bodansky units. Marked elevation of the alkaline phosphatase level is considered primarily the result of retention of the enzyme due to interference with biliary excretion,<sup>38</sup> since it occurs chiefly in cases of surgical jaundice, thus accounts for the absence of relation to liver cell damage. In the group with values between 4 and 10 units, medical jaundice predominated. This level might be explained by a lesser degree of bile flow interference or, as has been claimed, by an increased formation by the damaged liver cells.<sup>38, 41</sup>

Bromsulfalein retention (studied only in nonjaundiced patients) was related only to liver cell damage. Elevation of urinary urobilinogen was usually absent without liver damage. On the whole, however, in the applied approach they could not be correlated with each other since in the presence of biliary obstruction (intra or extrahepatic) urobilinogen may be absent from the urine despite marked liver cell damage. Stool urobilinogen being primarily an indicator of biliary excretion showed no relation to the studied phenomena.

The close relation of reduction of prothrombin to liver cell damage is due to the fact that in the absence of parenchymal damage the prothrombin time was invariably normal. However, the reverse did not hold true, that is, the prothrombin time was occasionally normal in spite of histologically recognizable cell damage. As generally accepted a pathologic prothrombin time is evidence of liver function impairment, provided other causes of hypoprothrombinemia such as poor vitamin K absorption due to obstructed bile flow or intestinal disorders are ruled out.

The reduction of the plasma vitamin A level showed a fair relation to liver cell damage in that when low values were found in liver disease the parenchymal cells almost constantly appeared damaged. Low vitamin A levels have been considered a test for impaired hepatic function.<sup>2, 40, 43</sup> Obviously the reduction can also be caused by other factors leading to endogenous vitamin A deficiency. This explains why in some instances without liver cell damage low vitamin A values were found.

The sedimentation rate revealed a correlation with periportal activity, probably because of the latter's inflammatory character. The correlation of the sedimentation rate with distorted reconstruction of the lobular pattern may stem from the fact that cases of active cirrhosis showing such reconstruction usually exhibit inflammatory periportal activity.

In general, the presented correlations may aid in the differential diagnosis of liver disease by a morphological evaluation of the function. An association between function tests and morphologic alterations, even without causative connection, is significant.

#### SUMMARY

In one hundred thirty patients (thirty five repeatedly studied) suffering from various liver diseases the histologic picture of the liver as seen in biopsy specimens obtained by aspiration or laparotomy was compared with the results of a series of liver function tests performed at the time of biopsy.

The incidence of seven morphologic phenomena (diffuse liver cell damage, focal necrosis, regeneration, distorted reconstruction, periportal activity, fatty metamorphosis, and Kupffer cell mobilization) was compared statistically with the results of each of different liver function tests.

A significant correlation was found between diffuse liver cell damage and albumin/globulin ratio, cephalin cholesterol flocculation, thymol turbidity, and bromsulphalein retention, a lesser degree with highly elevated serum bilirubin, reduced plasma vitamin A, increased prothrombin time and slightly elevated alkaline phosphatase. No correlations were elicited between parenchymal damage and total serum protein, total cholesterol, cholesterol ester ratio, urinary urobilinogen, stool urobilinogen, alkaline phosphatase in general (and markedly elevated alkaline phosphatase specifically), nonprotein nitrogen, or sedimentation rate.

Focal necrosis, in contrast to diffuse liver cell damage, was not associated with significant changes in liver function. The same was found with fatty metamorphosis.

Regeneration showed a correlation with increased thymol turbidity. Distorted reconstruction of the lobular pattern (as seen in cirrhosis) was related to cephalin cholesterol flocculation, thymol turbidity, and elevation of sedimentation rate. Kupffer cell activity appeared related to elevated serum bilirubin and pathologic albumin/globulin ratio.

These findings provided a basis for the morphologic evaluation of various liver function tests and a discussion of the functional significance of the aforementioned pathologic phenomena.

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# THE IMPORTANCE OF THE RATE OF DYE REMOVAL IN THE BROMSULFALEIN TEST OF LIVER FUNCTION

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FOLLOWING the introduction of bromsulfalein (BSP) retention as a test of liver function by Rosenthal and White<sup>1</sup> in 1925, numerous attempts have been made to improve the clinical method. Currently, the accepted procedure for the test is that recommended by Mateer and co-workers<sup>2</sup>. This procedure uses intravenously 5 mg of BSP per kilogram body weight and accepts 4 per cent retention in forty-five minutes as the upper limit of normal liver function. The BSP concentration in serum is determined by the method of Gaehler.

Several years ago MacDonald<sup>4</sup> published clinical evidence indicating the value of serial BSP determinations in the detection of liver damage. By taking blood samples at frequent intervals after the injection of 5 mg per kilogram of BSP intravenously he was able to detect liver damage not evident from a single forty-five minute blood sample. The study here reported was undertaken to compare the value of the fifteen-minute blood sample with the forty-five minute sample in the detection of liver damage.

## EXPERIMENTAL OBSERVATIONS

There is abundant evidence that intravenously injected BSP is not quantitatively excreted in the bile but is eliminated by other systems of the body, particularly the reticulo endothelial system<sup>5,6,7,8</sup>. After determining in preliminary experiments in dogs that 5, 10, and 20 mg per kilogram of BSP intravenously yielded reproducible and quantitatively similar curves, the effect of blocking the reticulo endothelial system with India ink was studied. Fig 1 records a typical experiment. It will be noted that, although within 48 hours after the injection of the India ink the forty-five minute serum BSP level was below 1 mg per cent (10 per cent retention), as long as 192 hours later the fifteen-minute serum concentration remained above the control level.

Following splenectomy (Fig 2) with removal of this portion of the reticulo endothelial system, there was a temporary increase in BSP retention. As illustrated in Fig 2, this retention was evident in the fifteen-minute serum sample two days after the forty-five minute sample had returned to control levels.

After obtaining a control BSP retention test using 5 mg per kilogram intravenously, hepatic damage was produced in rabbits by giving 0.5 ml per kilogram of carbon tetrachloride in corn oil via stomach tube. BSP retention was tested two days and ten days after the administration of carbon tetrachloride. Table I records the marked BSP retention that occurred in the fifteen minute serum sample long after the forty-five minute sample had returned to control levels.

The results of these experiments indicated that, when the reticulo endothelial system was compromised (India ink injection, splenectomy) or when

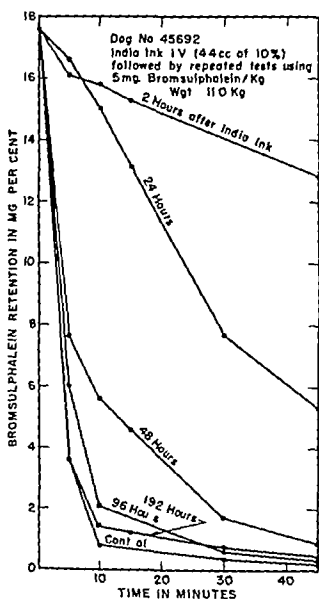


Fig 1

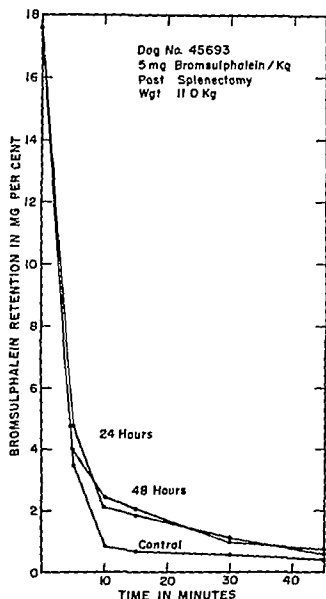


Fig 2

TABLE I PERCENTAGE OF BROMSULFALEIN RETENTION

RABBIT NO	CONTROL		TWO DAYS AFTER CCL <sub>4</sub>		TEN DAYS AFTER CCL <sub>4</sub>	
	15 MIN	45 MIN	15 MIN	45 MIN	15 MIN	45 MIN
59007	3	1	65	4	15	2
59049	1	0	71	3	9	1
59603	3	0	58	2	6	1
59606	4	1	54	2	45	12

hepatic change was produced by the oral administration of carbon tetrachloride the fifteen minute serum BSP sample reflected the change long after the forty five minute sample had become normal

#### CLINICAL OBSERVATIONS

BSP determinations using Gaebler's method<sup>2</sup> and 5 mg per kilogram doses in twenty individuals with normal liver function are recorded in Table II. Table III includes the BSP retention data from twenty one individuals hospitalized with miscellaneous disorders.

All the normal individuals (Table I) had a fifteen minute BSP serum concentration below 25 per cent. This finding agrees with the observations of MacDonald<sup>4</sup> who reported more than 25 per cent retention in fifteen minutes in only three of thirty eight patients. In Patients 1, 7, 8, 9 and 21 in Table III



TABLE II BROMSULFALEIN RETENTION IN INDIVIDUALS WITH NORMAL LIVER FUNCTION

PATIENT	DIAGNOSIS	SEX	AGE (yr)	BROMSULFALEIN RETENTION (%)	
				15 MIN	45 MIN
1	Upper respiratory infection	M	54	14	3
2	Multiple sclerosis	F	34	5	0
3	Hypothyroidism	F	38	22	3
4	Contusions of face	F	52	19	4
5	Pneumonia	M	54	14	3
6	Morphine addiction	M	54	20	4
7	CNS syphilis	M	55	5	2
8	Hypothyroidism	F	30	5	1
9	Pneumonia	M	37	18	2
10	Parkinsonism	M	67	23	4
11	Anal fissure	M	64	15	1
12	Tendon suture	M	38	13	2
13	Renal glycosuria	M	44	11	1
14	Skin graft	M	52	22	2
15	Finger amputation	M	26	22	6
16	Control (student)	M	31	14	2
17	Control (student)	M	24	18	3
18	Tonsillitis	M	20	17	3
19	Control (technician)	F	23	14	4
20	Control (technician)	F	23	16	4

(all of whom had some evidence of hepatic disease) the forty-five minute sample was within Mateer's upper normal limits of 4 per cent retention, although the fifteen-minute sample revealed more than 25 per cent BSP retention.<sup>2</sup> Attention is called particularly to Patient 21 with congestive heart failure. In our experience, increased BSP retention in the presence of congestive heart failure is likely to be more evident in the fifteen-minute than in the forty-five minute sample. The decrease in BSP retention that occurs with recovery from congestive heart failure, demonstrated here, has been repeatedly observed.

TABLE III BROMSULFALEIN RETENTION IN INDIVIDUALS HOSPITALIZED WITH MISCELLANEOUS DISORDERS

PATIENT	DIAGNOSIS	SEX	AGE (yr)	BROMSULFALEIN RETENTION (%)		COMMENTS
				15 MIN	45 MIN	
1	Goiter	M	44	29	1	BMR, + 22%
2	Hydrocele	M	55	37	18	Wine, quart daily for 10 yr
3	Hyperthyroidism	F	32	34	12	BMR, + 32%
4	Cirrhosis	M	70	52	15	Whiskey, quart daily for 4 mo
5	Bile duct carcinoma	M	69	32	27	Icterus index 40 units
6	Cirrhosis	M	60	30	18	Serum protein, 5.5 Gm %, ascite
7	Cirrhosis	M	60	36	4	Cephalin flocculation 3+
8	Pernicious anemia	M	58	32	1	RBC, 14 million
9	Carcinoma of rectum	M	69	27	3	Hepatomegaly
10	Alcoholism, diabetes	M	66	41	8	No hepatomegaly
11	Finger amputation	M	60	32	8	Formerly drank quart wine daily
12	Trophic ulcers	M	16	30	8	Spina bifida
13	Bronchiectasis	M	39	27	7	Post operative, lobectomy
14	Carcinomatosis	M	39	31	20	Primary site undermined
15	Hepatitis	F	39	77	49	Icterus index 30 units
16	Hepatitis	F	26	66	47	Icterus index 100 units
17	Hyperthyroidism	F	34	50	22	BMR, + 42%
18	Hyperthyroidism	F	44	42	12	BMR, + 28%
19	Gallstones	F	44	38	7	Asymptomatic
20	Cholecystitis	F	40	46	6	Subsiding, afebrile, no jaundice
21	Congestive heart failure	M	43	32	2	In severe failure, congestive hepatomegaly
				6	1	After recovery 6 wk later

TABLE IV CHANGES IN BSP RETENTION DURING HOSPITALIZATION FOR ALCOHOLISM

CASE	AGE	SEX	DURATION OF CHRONIC ALCOHOLISM	DURATION OF DRINKING BOUT IMMEDIATELY BEFORE HOSPITALIZATION	BROMSULFALFIN RETENTION ON ADMISSION IN PER CENT RETENTION		DURATION OF HOSPITALIZATION	BROMSULFALFIN RETENTION ON DISCHARGE IN PER CENT RETENTION		DIETOTHERAPY
					15 MIN	45 MIN		15 MIN	45 MIN	
1	46	F	10 yr	3 mo	42	4	2 wk	20	0	House diet without supplements
2	48	F	16 yr	4 wk	40	10	2 wk	30	0	High protein CHO diet with supplements
3	39	M	2 yr	2 wk	40	4	2 wk	20	3	High protein CHO diet with supplements
4	40	M	45 yr	2 wk	34	10	10 days	21	7	High protein CHO diet with supplements
5	48	M	20 yr	6 wk	34	10	3 wk	18	4	House diet without supplements
6	33	M	20 yr	3 wk	38	4	2 wk	18	2	High protein CHO diet with supplements
7	50	M	20 yr	18 days	32	2	1 wk	10	0	House diet without supplements
8	44	M	4 yr	1 yr	80	10	4 wk	30	6	High protein CHO diet with supplements
9	46	M	20 yr	4 wk	38	4	2 wk	18	0	High protein diet with supplements
10	40	F	8 yr	4 wk	60	30	2 wk	27	15	High protein CHO diet with supplements
11	48	M	20 yr	6 wk	42	40	2 wk	60	18	High protein CHO diet with supplements
12	33	F	14 yr	8 mo	90	80	3 wk	34	30	High protein CHO diet with supplements
13	5	M	20 yr	2 wk	36	12	3 wk	26	4	House diet without supplements
14	30	M	18 yr	6 mo	97	90	6 wk	40	22	High protein CHO diet with supplements
15	33	F	12 yr	4 mo	42	16	2 wk	18	4	House diet without supplements
16	40	M	20 yr	3 mo	52	14	1 wk	28	12	High protein CHO diet with supplements
17	62	M	18 yr	3 mo	54	16	2 wk	28	8	House diet without supplements

Table IV records the BSP retention of seventeen chronic alcoholics hospitalized for acute intoxication. At the time of admission to the hospital the BSP retention in forty five minutes was 4 per cent or under in five cases. However, the fifteen minute sample revealed 30 per cent or more BSP retention in every instance.

With abstinence from alcohol, and with sedation as necessary (chloral hydrate, paraldehyde) and an adequate dietary regimen there was a distinct decrease in BSP retention in every case. The decrease in BSP retention was particularly striking in the fifteen minute sample in Cases 1, 3, 4, 6, 10, and 13 which had exhibited only slight increases in BSP retention before the inception of therapy.

Six of these individuals (Cases 1, 5, 7, 13, 15, 17) received only the routine hospital diet without a high protein diet or vitamin supplements. The eleven other subjects received daily a high protein diet (120 Gm or more), 6 Gm of

choline dihydrogen citrate in divided doses, and 6 capsules each containing 110 mg ascorbic acid, "d" calcium pantothenate 9.5 mg, choline chloride 20.5 mg, folic acid 5.5 mg, inositol 54.5 mg, niacinamide 110 mg, pyridoxine HCl 10 mg, riboflavin 13.0 mg, thiamin HCl 16.5 mg, and liver powder 220.0 milligrams\*

In this experiment, improvement in liver function as evidenced by a decrease in BSP retention occurred both in those patients receiving the high protein diet with supplements and in those receiving the routine hospital diet without choline and vitamin supplements. Although the clinical impression obtained that the patients on the high protein, high vitamin regime responded more rapidly, no conclusive evidence in this regard was observed.

#### SUMMARY

These data indicate that sufficient additional information is obtained from the fifteen-minute serum sample in the BSP retention test to justify its use along with the forty-five minute sample.

It is suggested that with the BSP method used in this study 25 per cent retention in the fifteen-minute sample is probably the upper limit of normal. As noted by Mateer and associates,<sup>2</sup> 4 per cent retention marks the upper limit of normal in the forty-five minute sample.

The decrease in BSP retention following recovery from acute intoxication in a group of chronic alcoholics is reported. This decrease in BSP retention was not markedly different in patients receiving the routine hospital diet from those receiving a high protein diet with choline and vitamin supplements.

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\*The choline dihydrogen citrate and the vitamin capsules were supplied through the courtesy of Dr. Stanton M. Hardy of Lederle Laboratories Inc., New York, N. Y.

# THE EFFECT OF ORAL ADMINISTRATION OF CASEIN HYDROLYSATE ON THE TOTAL CIRCULATING PLASMA PROTEINS OF MAN

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NUMEROUS investigators report that diseases and physical injury can effect not only a decrease in plasma protein concentration but also a change in the composition of the plasma proteins<sup>1-6</sup>. The outstanding changes common to many of these situations are a decrease in the per cent of albumin and an increase in the per cent of alpha globulins such as are commonly observed in protein depletion<sup>7-9</sup>. Consequently, the changes in the plasma protein patterns in many situations may be due to protein depletion rather than to an inherent specificity of a given disease. Should this be the case in fact then the feeding of a suitable protein should result in a return toward normal of the plasma protein pattern without the necessity of effecting at the same time any significant change in the disease itself. Some selection of the protein may be necessary since it would appear that different proteins may cause regeneration of different plasma proteins to different degrees<sup>10-11</sup>.

The ability of a protein or a hydrolysate of a protein to stimulate the regeneration of various plasma proteins has some importance in itself, since hydrolysates have come into clinical use<sup>1-14</sup>. The present investigation is concerned with a study of the plasma protein pattern in a variety of clinical cases and with the ability of a casein hydrolysate\* used as the sole source of protein to cause restoration of abnormal patterns to normal.

## EXPERIMENTAL METHODS

Plasma volumes were determined by the method of Gregersen and Stewart<sup>15</sup> using Evans blue dye (T 1524). The total protein concentration of plasma was determined by the micro Kjeldahl method with selenium and copper sulfate as catalysts. For electrophoretic analysis of the plasma proteins about 10 ml of plasma were diluted with an equal volume of diethylbarbiturate buffer at pH 8.4 and ionic strength of 0.1 and then dialyzed against 2.0 liters of the same buffer for twenty-four hours in a cold room. The scanning technique and the method of resolution of Longsworth<sup>16</sup> were used.

*Chemical and Biologic Characteristics of the Hydrolysate Used*—The characteristics of the casein hydrolysate used in these studies were determined because a protein or protein hydrolysate would be expected to regenerate plasma protein only if it contains the essential amino acids in adequate proportions as evidenced by chemical analysis and by ability to support nitrogen balance

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\*The casein hydrolysate was supplied to us by E. R. Squibb & Sons, New York, N. Y.

and to promote growth of young animals at a low total dosage. The essential amino acid content of the hydrolysate is given in Table I. The streptogenin content, the nitrogen balance index as observed in dogs and the growth efficiency obtained in rats are given in Table II.

These data demonstrate that the hydrolysate used is equal to a high grade of edible casein in nutritive properties, casein itself being an adequate protein for most nutritive purposes. This view is also borne out by comparative feeding of casein and this hydrolysate to protein depleted rats<sup>21</sup> and by its ability to support nitrogen balance in man at a level of 0.18 to 0.20 Gm. nitrogen per kilogram per day.<sup>18</sup>

TABLE I ANALYSES OF THE TEN ESSENTIAL AMINO ACIDS AND CYSTINE AND TYROSINE IN A CASEIN HYDROLYSATE

AMINO ACIDS	PER CENT FOUND*	
	"AS IS" BASIS	H <sub>2</sub> O AND ASH FREE BASIS
Arginine†	2.9	3.2
Lysine†	6.7	7.3
Tryptophane†	1.1	1.2
Threonine†	4.3	4.7
Histidine†	2.7	2.9
Phenylalanine†	4.6	5.0
Valine†	5.9	6.4
Methionine†	2.7	2.9
Isoleucine†	5.9	6.4
Leucine†	8.7	9.5
Cystine‡	0.4	0.43
Tyrosine	2.8	3.0

The authors are indebted to Dr. R. D. Greene of E. R. Squibb & Sons for the determination of the amino acids.

\*The total nitrogen content is equal to 13.5 per cent of which 25 to 30 per cent is in the form of free amino nitrogen.

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TABLE II COMPARISON OF THE BIOLOGIC PROPERTIES OF CASEIN AND THE CASEIN HYDROLYSATE

TESTS	SUBSTANCE TESTED	
	CASEIN*	CASEIN HYDROLYSATE
Streptogenin (Units/Gm.)	4.5	4.5†
Nitrogen balance index	0.80	0.80‡
Growth efficiency	2.2	2.2§

\*The casein used was a high grade of edible casein from the Borden Company, New York.

†Woolley, D. W. J. Biol. Chem. 162, 383, 1946.

‡Allison, J. A. J. Nutrition 29, 413, 1945.

§We are indebted to Dr. A. Black of E. R. Squibb & Sons for making the growth efficiency tests.

## RESULTS

The determination of the ability of casein hydrolysate to promote plasma protein regeneration and the estimation of the type of plasma protein regenerated were made by feeding experiments wherein the casein hydrolysate was administered to two groups of hypoproteinememic patients, a total of twenty-eight subjects. Dextrimaltose was given so that the daily energy intake of the patients was at least 3,000 calories. The plasma protein concentration and electrophoretic

\*The authors are indebted to Dr. Paul R. Cannon and associates for the determination.

analyses were performed on samples of plasma taken before and after two to four weeks of administration. The patients in Group I as a whole were hypoproteinemic\* and not hypoalbuminemic, and the albumin globulin ratios were essentially normal. Therefore in order to study the effect of casein hydrolysate feeding their total circulating plasma proteins were determined before and after the hydrolysate feeding period. The patients in Group II were not only hypoproteinemic but also markedly hypoalbuminemic. The albumin globulin ratio of the plasma was considerably below normal in all patients. Hence the increase of albumin content following the administration of casein hydrolysate was marked. The other components of the plasma protein were also determined before and after the hydrolysate therapy.

*Group I The Increase of Total Circulating Proteins Following the Oral Administration of Casein Hydrolysate to Hypoproteinemic Patients*—Casein hydrolysate was fed to three patients after herniotomy at a low level of nitrogen intake (0.2 Gm. nitrogen per kilogram body weight per day) for about two weeks (Table III). These patients though in nitrogen equilibrium, were unable to increase their circulating plasma protein. Two other patients after herniotomy were fed at a moderate level of nitrogen intake (0.5 and 0.6 Gm. nitrogen per kilogram body weight per day). A pronounced increase of plasma proteins was observed in both patients. Subsequent studies used a high dosage level ranging from 0.6 to 1.0 Gm. nitrogen per kilogram body weight per day for periods ranging from ten days to seven weeks.

Total circulating proteins of fifteen patients characterized by different types of disease were determined before and after hydrolysate feeding (Table IV). An increase of at least 15 per cent in plasma protein was obtained in ten of the fifteen patients. An increase of somewhat more than 10 per cent the limit of accuracy of the estimation was observed in the remaining five. This increase involved both albumin and globulin fractions in all subjects but one. Patient An J. Hydrolysate feeding did not produce any consistent or significant changes in the per cent of any particular globulin fractions.

TABLE III DETERMINATION OF TOTAL CIRCULATING PLASMA PROTEINS OF PATIENTS AT DIFFERENT LEVELS OF NITROGEN INTAKE

PATIENT	NITROGEN DOSAGE (GM./KG./DAY)	NITROGEN GAIN (GM./KG./DAY)	DAYS OF TREATMENT	PER CENT GAIN (+) OR LOSS (-) OF TOTAL CIRCULATING PROTEIN	A/G*
La	0.2	0.0	12	10	1.08
Ka	0.2	0.06	13	5	1.13
Ma	0.2	-0.02	14	-4	-
Si	0.5	+0.13	18	+19	1.44
Be	0.6	+0.2	7	+18	1.04

\*Ratio of albumin to globulin before treatment as determined by the immunologic method

*Group II Changes of Plasma Patterns Following the Administration of Casein Hydrolysate to Hypoproteinemic and Hypoalbuminemic Patients*—Eleven patients with albumin ranging from 20 to 43 per cent of the total plasma protein were fed with hydrolysate at an intake of 0.6 to 1.0 Gm. nitrogen per

\*The term hypoproteinemia is used here to mean abnormally low in total circulating proteins which takes into account not only plasma protein concentration but also plasma volume.



TABLE V EFFECT OF ORAL FEEDING OF CASEIN HYDROLYSATE ON PLASMA PROTEIN OF HALOALBUMINEMIC PATIENTS

PATIENT	DAYS OF TREATMENT	PLASMA PROTEIN (GM/100 ML)	PLASMA VOLUME (ML)	ALBUMIN/ GLOBULIN	PLASMA PROTEIN COMPONENTS (GM %)						DIAGNOSIS
					ALBUMIN	ALPHA GLOBULIN	ALPHA GLOBULIN	BETA GLOBULIN	FIBRINOGEN	GAMMA GLOBULIN	
Eg†	0	7.40	--	0.35	1.02	0.59	1.11	0.52	0.44	2.81	Pemphigus
	13	6.73	---	0.49	2.23	0.68	0.93	0.34	0.47	2.09	
	43	7.68	---	0.67	3.07	0.46	1.00	0.69	0.38	2.07	
Yo	0	4.62	--	0.23	0.02	0.60	1.43	0.55	0.33	0.35	Cancer
	17	6.52	--	0.59	2.41	0.39	0.89	1.04	0.63	0.98	
	20	7.00	--	0.67	2.40	0.56	0.77	1.19	0.77	0.91	
Da	0	5.36	3030	0.75	2.30	0.54	0.80	0.64	0.21	0.86	Malnutrition
	23	7.01	3432	1.27	3.93	0.35	0.84	0.49	0.28	1.12	
	0	6.16	2640	0.49	2.23	0.47	1.28	1.19	0.27	1.33	Pulmonary tuberculosis
Tot	11	6.72	2498	1.13	3.06	0.34	0.20	0.67	0.67	1.28	
	68	6.40	2462	1.08	3.33	0.42	0.38	0.58	0.64	1.02	
Su	0	6.02	3042	0.64	2.32	0.34	0.90	0.60	0.42	1.20	Malnutrition
	43	6.01	3940	0.73	2.59	0.60	1.14	0.84	0.06	0.78	
	0	5.99	--	0.47	1.88	0.33	1.06	0.71	0.24	1.47	Diabetes mellitus
Po	60	5.00	--	0.82	3.60	0.32	1.04	0.72	0.12	2.00	

The casein hydrolysate used was Casein Hydrolysate (Squibb)

†The casein hydrolysate used was Amigen Meal Johnson &amp; Company Evansville, Ind





kilogram body weight per day. The patients in this series were divided into two groups. Subgroup A six subjects responded to the hydrolysate therapy and subgroup B five subjects failed to respond to the casein hydrolysate feeding; the latter were all in the terminal stages of their diseases.

*Subgroup A.* The results given in Table V demonstrate that administration of this hydrolysate brought about a rapid increase in plasma protein concentration or in plasma volume in all patients but one (Patient Lo). The plasma volume of Patient Eg was not determined; therefore its change if any is not known. The albumin content in terms of gram per cent was increased in all cases. As would be expected, the increase was most marked where hypoalbuminemia and hypoproteinemia were most severe, that is where albumin globulin ratio and plasma protein concentration were the lowest.

The response of Patient Lo to the hydrolysate therapy is of particular interest because there was neither increase in protein concentration nor in plasma volume after sixty-eight days of the hydrolysate therapy. Nevertheless there was a very significant increase in albumin globulin ratio. The increase in albumin therefore was made at the expense of the globulins.

*Subgroup B.* Four hypoalbuminemic patients with albumin content of 30 per cent or less were fed with casein hydrolysates at a level of as much as 0.6 Gm nitrogen per kilogram body weight per day for about two weeks or longer. During this period the patients were in positive nitrogen balance and retained as much as 0.4 Gm nitrogen per kilogram body weight per day. However in spite of the large nitrogen retention the pattern of the plasma proteins of these patients failed to approach normal (Table VI). The albumin globulin ratio remained low and there was no significant change in the total circulating albumin or globulins. No one of these four patients who did not respond to the therapy survived for any great length of time following these observations. The results may indicate that during the advanced stages of certain diseases patients may be unable to utilize an otherwise adequate mixture of amino acids and polypeptides for the synthesis of plasma proteins.

#### DISCUSSION

The patients used in this study reacted much the same as do experimental animals to gradual protein depletion. Consequently results are consistent with the belief that of the systemic manifestations of diverse diseases the abnormalities observed in the pattern of the plasma proteins and in their amount are in part the results of a general protein deficiency.

A casein hydrolysate which contains all the essential amino acids and high biologic values as determined in laboratory animals and in man was administered to patients to correct the abnormal plasma patterns. The patients used constituted a rather heterogeneous group. They were characterized by different degrees of hypoproteinemia and by different types of disease; in addition the period of hydrolysate therapy was not always uniform. However in spite of these variations the results are sufficiently striking to indicate that in general the administration of a suitable casein hydrolysate at a sufficiently high level to hypoalbuminemic patients results initially in a rapid increase in plasma

albumin content. Such a result is not to be expected in the terminal phases of certain diseases. When the hypoalbuminemia is not severe, the feeding of casein hydrolysate produces an increase of both plasma albumin and of plasma globulin. This effect on the globulin fractions may have special significance because of the possible relationship of certain globulins and certain of the so-called vital functions of the body related to the synthesis of certain of the hormones, the enzymes and the antibodies.

The failure of some patients to regenerate either albumin or globulins following casein hydrolysate therapy in spite of positive nitrogen balance is of interest. This fact is in line with other information which indicates that plasma proteins are not as readily synthesized as some other tissue proteins.

#### SUMMARY

Casein hydrolysate has been given to twenty-eight hypoproteinemic and hypoalbuminemic patients with a variety of diseases. It promotes the regeneration of both the plasma albumin and globulin fractions. In six cases of severe hypoalbuminemia, the albumin deficiency was corrected rapidly. Five hypoproteinemic patients at the terminal stages of their diseases were able to utilize the hydrolysate to maintain a positive nitrogen balance but not to regenerate plasma proteins.

These results are consistent with the belief that diverse medical and surgical conditions are characterized by varying degrees of protein depletion which can be corrected by large amounts of casein hydrolysate.

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# STUDIES ON THE MINIMUM PROTEIN REQUIREMENTS OF ADULT DOGS

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THE protein requirements of adult animals including man have been investigated almost exclusively by nitrogen balance studies. This is a reasonable method since only the losses through the skin and those involved in the growth of hair and skin are not taken into account. These are undoubtedly small. However, little work has been directed toward the determination of the proper conditions under which the studies should be made. It is known that when an animal is fed a low protein diet the urinary nitrogen falls rapidly during the first few days and continues to fall for a considerable period, but at a decreasing rate. It seems obvious that the amount of protein required to balance these losses will depend upon the time the studies are made, or the degree of nitrogen depletion of the subject.

The fundamental studies of Terroine and co-workers,<sup>1-3</sup> Sorg-Matter,<sup>2</sup> Ashworth and Brody,<sup>4</sup> and Smuts<sup>5</sup> have shown that eventually the nitrogen excretion reaches a minimum which is closely correlated with the basal metabolism of the subject. This minimum may be considered the endogenous level of nitrogen metabolism, and corresponds to 1.4 to 2.0 mg of nitrogen per basal calorie. Since this appears to be minimum nitrogen excretion, it is reasonably certain that the minimum nitrogen requirement cannot be below these levels. For an individual with a basal metabolism of 1,500 calories per day, the minimum daily protein requirement would thus be between 13 and 19 Gm, assuming complete utilization. Most proteins are of course not completely utilized and the minimum requirements are above these levels. The amount above may be calculated from a consideration of the degree of digestibility and the biologic value of the protein fed. The recent studies of Bickei, Mitchell, and Kinsman<sup>6</sup> and Hegsted, Tsongas, Abbott, and Stare indicate that the protein requirement of human adults is in this range, that is, endogenous requirement plus corrections for digestibility and biologic value, and have yielded minimum values considerably below the previously accepted figures.

However, it must be recognized that although nitrogen balance is an apparently adequate criterion of whether an animal is being maintained with regard to nitrogen, it tells nothing of the condition of the animal thus being maintained. It is not impossible that continued maintenance of an animal in a depleted state may lead to serious consequences. From the studies of Addis and associates<sup>7-10</sup> it would appear that animals depleted to endogenous levels of nitrogen excretion would have severely depleted livers, and Elman and co-workers<sup>11</sup> have shown that plasma protein levels begin to fall soon after low

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## LIVERS FROM NORMAL AND EXPERIMENTAL DOGS

LIVER NITROGEN					LIVER LIPIDS (% DRY WT.)	CHOLINE (MG./100 GM. DRY WT.)	GLYCOGEN (% DRY WT.)
TOTAL (GM.)	% FRESH WT.	% DRY WT.	MG./KG. BODY WT.	MG./GM. NONLIPID NONGLY- COGEN LIVER			
0.557	4.090	13.11	1.422	-	8.3	-	-
1.003	3.363	14.69	0.880	-	17.6	-	-
16.348	3.270	11.55	0.910	-	-	-	-
17.844	2.974	10.05	0.940	-	-	-	-
19.036	3.461	11.69	0.710	-	-	-	-
9.749	1.457	5.39	0.670	102.6	8.9	602.3	38.6
9.69	0.080	0.74	0.630	99.6	12.3	64.2	13.4
9.571	2.138	6.75	0.640	132.6	5.1	266.5	43.9
9.949	0.234	1.20	0.83	138.9	13.7	511.2	34.4
6.63	1.68	5.70	0.87	113.0	10.0	-	31.0

chloroform. The chloroform extractable material was dried and weighed. The choline content was determined from another sample after grinding with anhydrous sodium sulfate and extraction with methanol by a modification of the remeckate method.<sup>18</sup>

The liver samples for glycogen determination were immediately sliced and dropped into a Pyrex tube of known weight containing a known amount of 30 per cent potassium hydroxide. The glycogen content was determined by the method of Good, Kramer and Somogyi.<sup>19</sup>

Thin slices (3 to 5 mm.) of all livers were taken at random from any lobe and fixed in formaldehyde-formol-alcohol-picric acid solution of Rossman and Zenker or in fixative sections were prepared by the paraffin or celloidin methods and stained with hematoxylin and eosin or in methylene blue and by the Best carmine method for glycogen. Frozen sections were stained with Sudan IV.

## RESULTS

The combined data on nitrogen balance, body weight, nitrogen and calories consumed, blood constituents, and body fluids are presented in Figs. 1 to 6. All dogs received 666 protein at comparable levels for thirty days after the depletion period. At this time the changes in dietary regime indicated in the illustrations and in Table I were made. The results on egg protein will be discussed first.

**Nitrogen Balance and Body Weight.**—Nitrogen excretion studies were begun after the dogs had received the nitrogen free diet for ten days. At this time the urinary nitrogen was clearly not at minimum levels since, with the exception of Dog 6, all the animals showed a stepwise reduction in nitrogen excretion in

subsequent periods. It is of interest that they showed this drop in nitrogen excretion even though egg protein was added to the diet. This result is in agreement with those of Miller,<sup>20</sup> Brush and co-workers,<sup>21</sup> and Allison and associates.<sup>2</sup> As has been discussed by these authors, this gives to egg protein a biologic value above 100. It is presumed that the animals would have reached this low level of nitrogen excretion eventually if continued on the nitrogen-free diet. Most of the dogs went into a slight positive balance eventually. Meanwhile the body weight increased slightly. It was the main purpose of the experiment to study the physiologic changes during this period.

*Plasma Volume and Plasma Proteins*—Prior to depletion, the figures obtained for plasma volume, 41.2 to 60.7 cc per kilogram, total plasma proteins, 6.3 to 8.28 per cent, and total circulating plasma protein, 2.6 to 4.0 Gm per

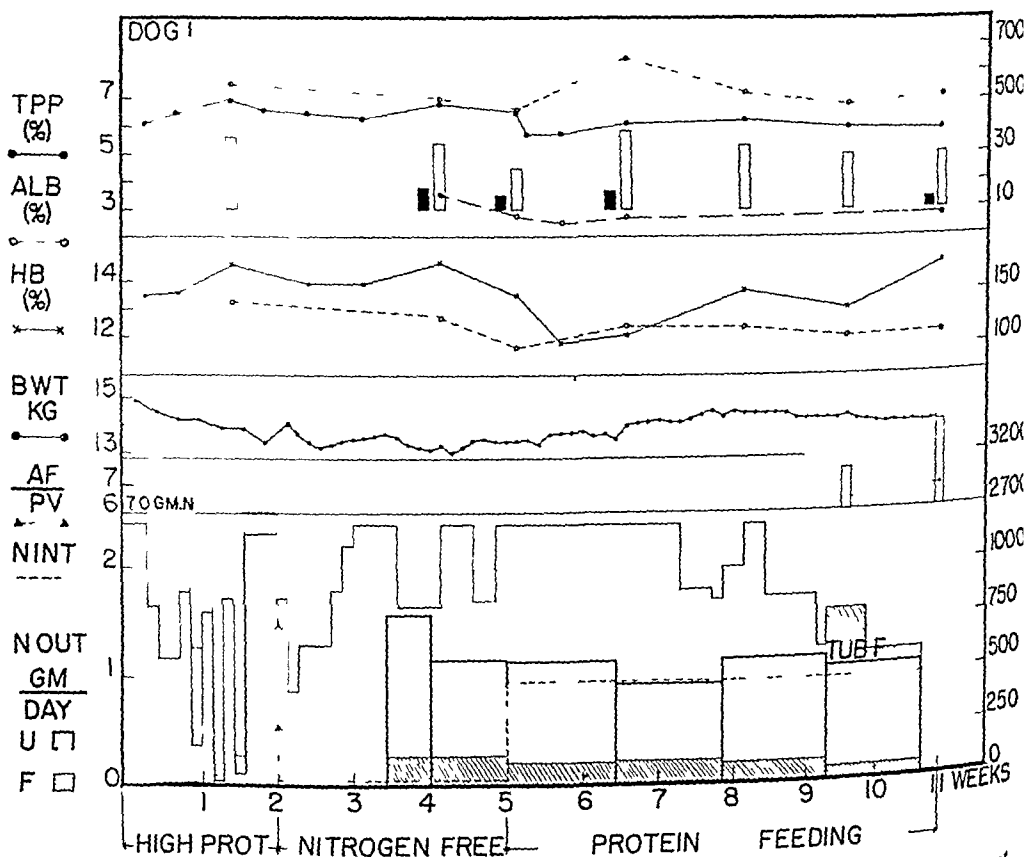


Fig 1—This figure summarizes the data obtained on Dog 1. TPP % Percentage of total protein in plasma. ALB % percentage of albumin in plasma. HB % per cent hemoglobin. BWT body weight in kilograms. AF/PV ratio of available fluid to plasma volume. NINT nitrogen intake grams per day. NOUT total nitrogen output grams per day. U urinary nitrogen and F fecal nitrogen. PV cc. plasma volume in cubic centimeters. TCPP Gm. total circulating plasma protein in grams. T ALB Gm total circulating albumin in grams. T CHB Gm total circulating hemoglobin in grams. AF cc available fluid volume in cubic centimeters. CAL calorie intake per day.

During the ninth week tube feeding (TUB F) was resorted to to maintain nitrogen intake and partial caloric intake.



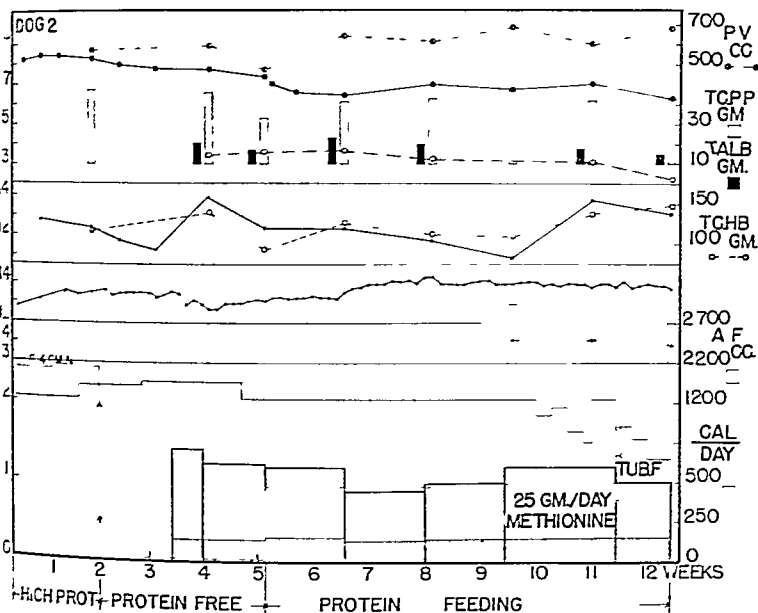


Fig. 2.—Data obtained on Dog 2. See Fig. 1 for interpretation.

Electrograms are normal when compared with those reported in the literature.<sup>1-3,2</sup> During the depletion period all of these decreased somewhat. This is of considerable interest since it indicates that there is a fall in plasma proteins before the minimum nitrogen excretion is reached. When egg protein was added to the diet all of the dogs with the possible exception of Dog 4 showed a favorable response in total plasma protein. In most dogs this was shown by an increase in plasma volume while the percentage of protein remained essentially constant. In Dogs 4 and 7 the concentration increased somewhat with less marked or no change in plasma volume. The significance of the two types of response is not clear.

During the rest of the periods the level of protein was apparently sufficient to maintain both plasma volume and protein concentration. There is no evidence of a sustained fall indicating further protein depletion. Raising the level of nitrogen intake of Dogs 3 and 7 by 20 per cent may have caused a slight response. Although in Dog 3 the control level of total plasma protein had been reached before the change was made. The last observation on this dog is probably complicated by the failure to maintain a maximum calorie intake. The same is certainly true for Dog 1.

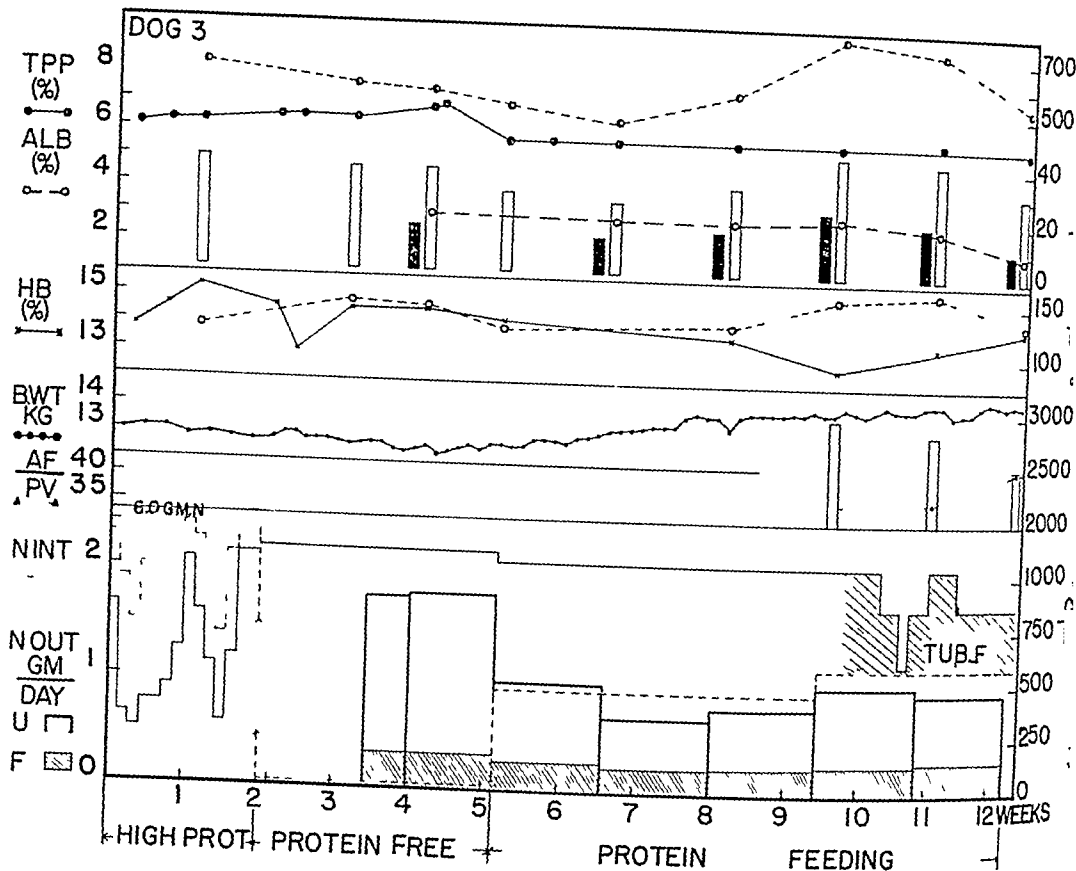


Fig 3—Data obtained on Dog 3 See Fig 1 for interpretation

**Hemoglobin**—Almost all of the dogs showed some loss of hemoglobin during the depletion period, a total of about 10 Gm per dog. With the exception of Dog 6, which was in relatively strong negative nitrogen balance throughout the study, there is little or no evidence of a progressively developing anemia after egg protein was fed. The percentage concentration of hemoglobin showed marked changes, especially with the changes in diet. The total circulating hemoglobin in spite of the fact that it is a calculated figure, appears to be much more useful in studies of this type in evaluating the nutritional status. The fact that the hemoglobin concentration changes were chiefly the result of the changes in blood volume is apparent from the figures, and the constancy of the total circulating hemoglobin lends considerable support to the correctness, at least relatively, of our figures for plasma volume.

**Available Fluid Volume**—Unfortunately, available fluid volumes were not determined early in the study, and some of the last values obtained may have been complicated by low calorie intakes. However, after the depletion period and thirty days on the low nitrogen regime the available fluid volume varied from 198 to 237 and the plasma volume from 33.5 to 55.4 cc per kilogram of body weight. The ratio of available fluid to plasma volume varied from 3.8 to

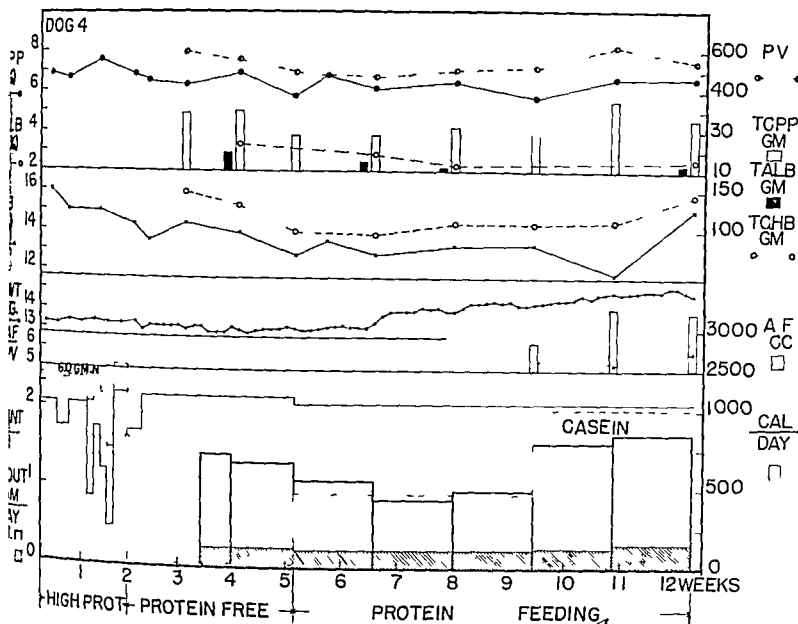


Fig 4—Data obtained on Dog 4. See Fig 1 for interpretation.

68 It is of interest that Dogs 1 and 6 which were in the strongest negative nitrogen balance showed the highest ratios 6.4 to 6.8. The values for the other dogs were in the normal range 3.2 to 3.9. The significance of later values for available fluid is problematical because of the failure of appetite in three dogs. This left only one dog receiving casein protein (at 20 per cent higher level than at the beginning). There appeared to be a steady increase in available fluid volume and ratio although plasma volume was constant. The dog was in slight positive balance after a considerable period of negative balance. Nevertheless this probably represented a continuing tendency toward edema. The opposite trend in Dog 6 was seen when nitrogen balance improved.

**Methionine Supplementation**—Methionine was added to the diet of two dogs because of reports that this amino acid is effective in conserving body protein.<sup>22</sup> Dog 6 responded as expected. The urinary nitrogen fell markedly so that the dog was in balance and this was maintained in the following period even though the level of casein protein was decreased below that used during most of the study. A response in the circulating proteins was accompanied by an increase in plasma volume. Both the total hemoglobin and the hemoglobin content increased. The ratio of available fluid to plasma volume fell. In Dog 2

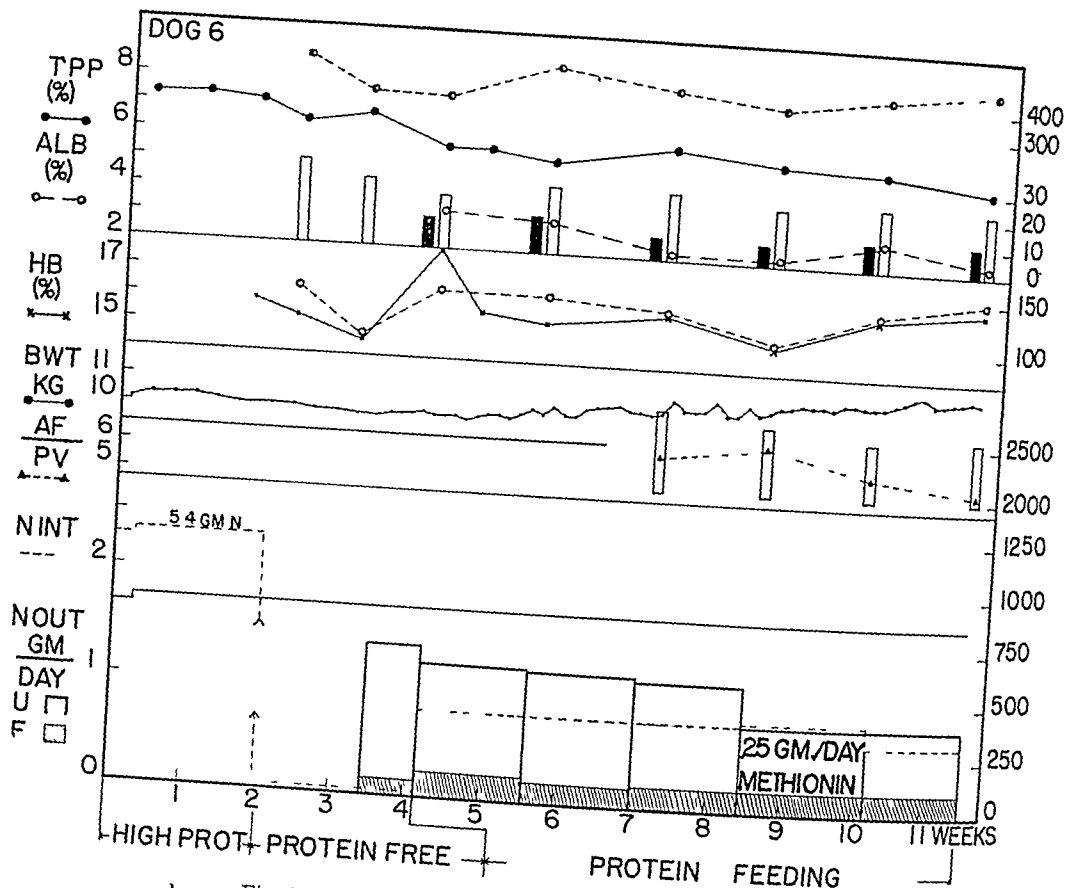


Fig 5—Data obtained on Dog 6 See Fig 1 for interpretation

the nitrogen balance became more negative. If the changes in plasma proteins and plasma volume are significant, they are opposite to those of Dog 6. The ratio of available fluid to plasma volume was maintained by a slight drop in available fluid. It should be noted that the ratio in Dog 2 was much lower than in Dog 6. There was, however, as in Dog 2, a favorable response in hemoglobin level and total hemoglobin.

It has been reported that the response to methionine is less marked after severe nitrogen depletion.<sup>20</sup> The data on nitrogen balance and total circulating protein indicate that Dog 6 was probably the more severely depleted. Thus the better response in Dog 6 was contrary to expectation. However, Dog 2 had previously shown a drop in urinary nitrogen to the endogenous level when given egg protein, while Dog 6 continued in strong negative balance. This may explain the difference in the two dogs at the time methionine was given, but the reason for the failure of Dog 6 to respond previously to egg protein remains unexplained.

**Casein Supplementation**—Dog 4 was given a relatively high level of casein supplying 2 Gm of nitrogen per day. This was sufficient to throw the dog in strong positive balance. Coincident with this response, all of the values for

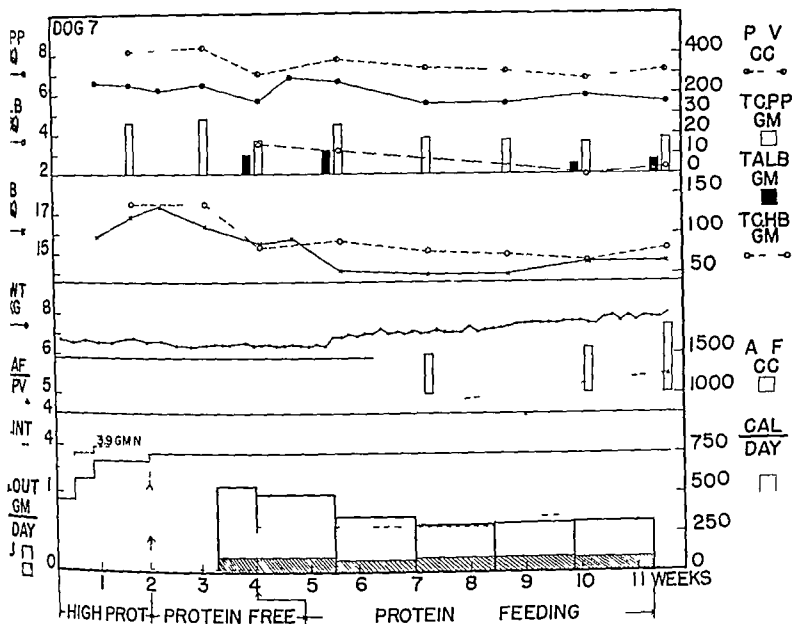


Fig 6—Data obtained on Dog 7 See Fig 1 for interpretation

plasma proteins, plasma volume, hemoglobin and available fluid increased. The ratio AF/PV remained constant between 5 and 6. It may be worth while to call attention to the changes in total circulating protein and plasma volume since this type of response was also observed in some of the dogs when they received protein previously. There appeared to be an increase in total protein as the primary response, and the changes in plasma volume followed this. In the next period the plasma protein apparently redistributed itself to body tissues. The total protein fell somewhat together with the plasma volume but not in proportion since the concentration of protein remained slightly higher than previously.

**Liver Analyses**—The results for moisture, fat, glycogen and choline are shown in Table I. The results obtained on the five supposedly normal dogs included two which had been given the purified diet containing 30 per cent skim milk powder for thirty days before they were killed are also presented. All animals had livers of normal fat and water content.<sup>30, 31</sup> The glycogen and choline varied greatly. The loss of protein of liver has been recognized as an early sign of fasting or protein deficiency.<sup>8, 10</sup> Kosterlitz and Campbell<sup>32</sup> found that the losses in protein, phospholipid and nucleic acid during fasting and in protein deficiency represent a loss of liver cytoplasm. They found that

this loss of liver cytoplasm could be expressed by a curve containing an exponential and linear component. The linear decrease is thought to be a measure of endogenous metabolism, while the exponential decrease is probably due to loss of labile liver cytoplasm. These data suggest that the determination of liver nitrogen may be a simple and accurate index in experimental animals of the nutritional status with regard to protein. In Table I the nitrogen content of the livers has been expressed in several ways. If the nitrogen per gram of nonglycogen nontat liver solids is plotted against the average nitrogen intake of the

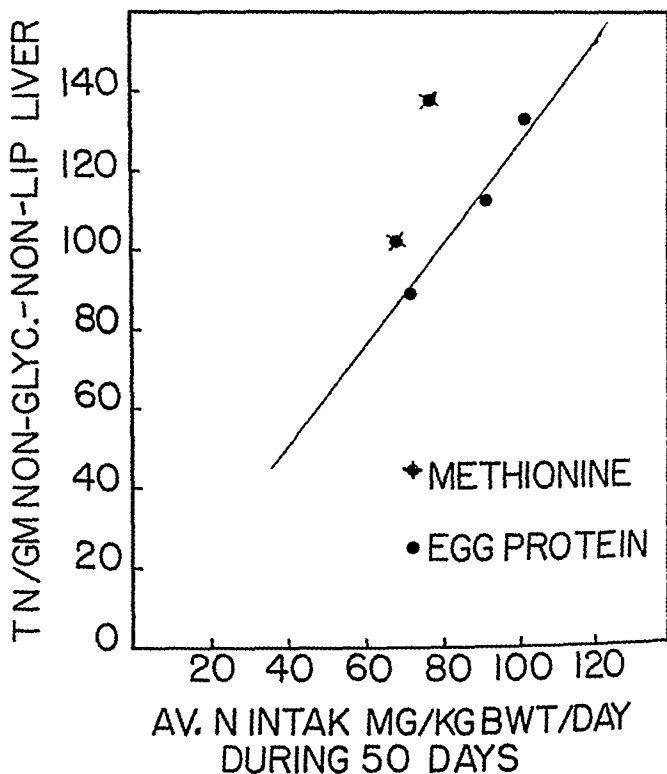


Fig 7—Diagram showing the relation between the average nitrogen intake milligrams per kilogram of body weight per day during the experimental period and the total liver nitrogen expressed as milligrams of nitrogen per gram of nonglycogen nonlipid liver

dog during the entire study (including the supplements at the end of the experiment), there appears to be practically a straight-line relationship for three of the animals (Fig 7). The two which fall above the line are those which received methionine supplements. These results agree with those recently reported by Brush and co-workers<sup>21</sup> and indicate a shift of protein toward the liver. It is interesting that this happened in Dog 2 although there was no apparent improvement in nitrogen balance.

The liver nitrogen contents per kilogram of body weight of our dogs are compared with various values taken from the literature in Table II. It is evident that on this basis our animals are considerably below well-fed animals and above severely depleted animals.

TABLE II. EFFECT OF DIET UPON THE NITROGEN CONTENT OF DOG LIVERS

SOURCE	DOG NO	DIETARY CONDITIONS	AVERAGE LIVER NITROGEN (GM /KG BODY WEIGHT)
Grand <sup>24</sup>	6	133.366 Gm N per kg body weight per day for 20 or 35 days	1.06
	4	Fasting for 13-19 days	0.57
Pughese <sup>3</sup>	2	Normal	0.91
	2	Fasting for 20-24 days	0.60
	2	250-300 c.c. of milk given daily for 4 days after 28-30 days' fast	0.81
Authors	4	2 mg N per basal caloric from egg protein for 40 or 50 days following 2-3 weeks on nitrogen free diet	0.72
	2	Same as Dog 4, plus methionine for 10 days	0.75
	2	Normal, high protein diet for 30-40 days	1.15
	3	Normal dog chow and hormone ment	0.89

*Pathology—*

*Gross* Except for a dark blue green color imparted by the Evans blue dye to the liver, no striking changes were noted. All livers were smooth and the capsules glistened. The borders were sharp. Although all of the livers were firm, there was no increase in resistance to section. The weights of all livers are recorded and compared with body weight in Table I. The other organs showed no significant changes. The livers of the five normal dogs also showed no significant change.

*Microscopic* The changes found in the livers of experimental animals 2, 3, 4, 6, and 7 differed only quantitatively, in detail and were similar to those described by Elman and Heifetz<sup>30</sup> in dogs on protein deficient diets.

There was marked swelling of liver cells and cell membrane was well defined and intensely stained. So extensive was the swelling of liver cells that the sinusoids were largely obliterated. Most striking of all changes was the marked rarefaction of cytoplasm which was represented by a few eosinophilic granules of the cytoplasm identified as ribonucleic acid by various techniques (see review by Greenstein<sup>32</sup>).

The liver cell nuclei were uniformly small, central and round or oval with a scant amount of fine, evenly dispersed chromatin material and prominent, often multiple nucleoli. In many instances binucleated cells were seen and in one animal (Dog 4) many of the nuclei were hyperchromatic.

In most livers, the Kupffer cells were unusually prominent because of coarse dark chromatin granules in their nuclei and fine brown pigment granules in the cytoplasm.

The changes in liver cells described affected the entire parenchyma but were most severe in the portal areas. In some of the central areas there was more intense staining and a few identifiable sinusoids indicating less rarefaction of cytoplasm and less swelling of the cells.

The livers of all experimental animals stained intensely for glycogen and were not distinguishable, one from another on this basis. The liver of only one of the control dogs fed on a high protein diet showed any appreciable amount of

glycogen by histochemical examination and even in this instance the amount was estimated to be less than half that found in the experimental animals. It appeared to be equally distributed between the portal and central areas, with considerably less present in the mid-zonal region. In the five normal dogs examined, only occasional patchy areas of glycogen were encountered. The three of these dogs which received chow and meat had been fasted sixteen to twenty hours, but the two receiving the high protein diet were killed at the same interval after feeding as the experimental animals. The significance of this apparent increase in liver glycogen in protein-depleted dogs is unknown.

Although stainable fat was present in appreciable quantities in the cytoplasm of the cells of the bile duct epithelium in the livers of all experimental animals, and to a lesser extent in the control and stock animals, none was found in the liver parenchyma of any animal. It may be concluded that the choline provided in the diet and the methionine fed as such or as protein was sufficient to prevent the development of fatty livers. The morphologic changes described as occurring in the livers of the experimental Dogs 2, 3, 4, 6, and 7 are probably wholly the result of protein deficiency. The accumulation of fat in the livers of protein deficient dogs described by Elman & Heifitz<sup>30</sup> may be due in part to choline deficiency.

*Appetite*—Of the six dogs started in this study, three showed marked failure of appetite toward the end of the study. This undoubtedly has had some effect upon the analytic values obtained, although it is not evident what this effect may be. There is also no certainty that the failure of appetite is specifically due to protein deficiency, although we consider this a likely possibility. The nitrogen intake of the dogs was maintained by tube feeding during this period, but it was impossible to maintain the caloric intake.

#### DISCUSSION

We have attempted to evaluate the adequacy of a protein level thought to be near a theoretical minimum, which would correspond to about 20 Gm in an average human adult. Numerous questions still remain unanswered and the study is being continued, but the present results are instructive. For the reasons discussed in the introduction, the animals were partially depleted of protein prior to being given the low level of protein. During this depletion period the data show that from 96 to 230 Gm of body protein were lost. This was accompanied by a loss of 9.5 to 13.5 Gm of circulating plasma protein. The percentage of plasma protein was also decreased slightly, but, without prior knowledge of the plasma levels, would be considered to be in the normal range. *These changes occurred before a minimum level of urinary nitrogen excretion was reached.* In our opinion the data on urinary nitrogen excretion and plasma proteins alone would not be considered indicative of severe protein depletion.

During the next four to six weeks when egg protein was fed, all of the analytic data indicate that the animals were maintained in this condition. The general average indicates that nitrogen balance was achieved, and the other constituents measured show no consistent deterioration. The changes during



the period on egg protein are summarized in Table III. However, we interpret the microscopic findings, swelling of the liver cells with mild rarefaction of the cytoplasm as indicating relatively severe liver depletion. Since the animals appeared to have been maintained, this degree of depletion was probably achieved during the period on the nitrogen free diet rather than while the egg protein was being fed. It is impossible to state how severe this depletion was or how serious the consequences were for the animal but the livers of these animals could not be considered normal. Thus we conclude that the animals which had not been depleted to an endogenous or minimum level of nitrogen excretion were nevertheless too severely depleted to be considered normal and that this base line is too severe to be used for studies which are to be interpreted in terms of normal animals. It seems further apparent that a barely detectable loss of plasma protein has reflected relatively severe liver depletion and that plasma protein determinations are insufficiently sensitive to evaluate such changes.

TABLE III ANALYTIC DATA OBTAINED AT THE END OF THE DEPLETION PERIOD COMPARED WITH THE VALUES AFTER APPROXIMATELY FOUR WEEKS ON THE DIET CONTAINING 2 MG NITROGEN PER BASAL CALORIE

DOG	PV (CC)	PP (GM %)	TCA (GM)	Alb (%)	TCA (GM)	Hb (%)	Hc (%)	TCH (GM)	BV (CC)	BWT (KG)
1 A	450	5.74	25.9	2.76	12.4	13.5	30.8	101	747	13.4
B	480	6.05	29.0	(2.60)	(12.7)	13.0	38.4	101	779	14.3
2 A	416	7.02	33.4	3.66	17.2	12.4	36.6	93	750	13.4
B	690	6.77	46.7	(3.00)	(20.7)	10.9	32.1	110	1016	14.0
3 A	504	5.76	29.0	(2.20)	(11.1)	14.2	41.8	123	867	12.7
B	476	5.76	44.7	3.10	24.1	12.6	37.2	155	1230	14.2
4 A	496	5.76	28.7	(2.30)	(11.4)	12.8	37.2	101	789	13.1
B	514	5.67	29.1	(2.10)	(10.8)	13.4	39.2	112	840	14.8
5 A	360	5.76	20.7	2.42	12.3	18.1	53.4	131	771	10.0
B	310	5.61	21.1	2.30	8.0	15.0	44.3	101	667	11.0
6 A	300	5.16	17.2	3.53	10.6	15.5	45.7	86	552	6.5
B	314	5.58	17.7	(2.20)	(6.9)	14.0	41.3	75	535	7.6
Average, all dogs										
A	431	5.91	25.8	2.96	12.8	14.4	42.4	105	746	11.5
B	514	5.92	31.4	2.56	13.4	13.1	38.8	109	846	12.6

PV, plasma volume; PP, plasma protein; TCA, total circulating albumin; Hb, hemoglobin; TCP, total circulating proteins; Alb, albumin; BV, blood volume; Hc, hematocrit; TCH, total circulating hemoglobin.

A, End of depletion; B, thirty days on egg protein. The figures in parentheses are estimated from determinations obtained a few days before and after the desired date.

The fact that appetite failed in most of these animals is a serious consequence. One cannot rule out possible deficiencies of other dietary factors as the causes of this, but dogs have been maintained upon a similar diet containing purified casein for long periods. It is not impossible that the low protein diets may change an animal's requirement for other nutrients but until this is shown we feel we must consider the loss of appetite as a symptom of protein deficiency. This has been observed in other studies on dogs to such an extent as to preclude

completion of the work.<sup>37</sup> Frazier and co-workers<sup>38</sup> have emphasized the immediate effect on appetite of acute amino acid deficiencies. It has been suggested that this is a protective mechanism and that the other amino acids may actually be detrimental in the absence of an essential one. It is also possible that calorie intake itself may be detrimental in this sense since this undoubtedly necessitates considerable work on the part of the liver. However, we have the impression from work during the past several years on low protein diets that the dog and rat are hardly comparable with regard to appetite. Although the food intake of protein-deficient rats is reduced, we have seldom seen a complete failure of appetite such as frequently occurs in dogs, and with rats this usually only has been seen with very severely depleted animals. In dogs, on the other hand, this may be the first suggestion that the diet is unfavorable. In this respect the dog is probably more nearly like human beings. Regardless of whether or not the reduction of food intake is considered a protective mechanism, the vicious cycle of mild deficiency  $\rightarrow$  loss of appetite  $\rightarrow$  severe deficiency is indeed vicious if it reaches the stage where the animal fails to eat an adequate diet when offered.

The differences in the response of the plasma proteins to the feeding of protein were interesting. If the time intervals between determinations had been shorter, it seems likely that the responses might have been more uniform. In most of the animals the response was in plasma volume rather than in the percent of plasma protein. When plasma protein was increased, this generally appeared to be redistributed to other tissues. Thus the priority seems to be for the maintenance of blood volume and for other tissues rather than for an increase in the concentration of the plasma proteins. If one could determine that level of protein intake at which the plasma levels begin to rise, indicating adequate plasma volumes and flow of protein to less essential systems, this might be a true minimum requirement. However, as mentioned earlier, it is not likely that the determination of plasma proteins would be sufficiently sensitive to identify this point with any assurance.

In a previous discussion,<sup>39</sup> one of us has pointed out that the effect of methionine or egg protein supplementation in lowering the excretion of urinary nitrogen appears to be a lowering of urinary nitrogen to endogenous levels without serious nitrogen depletion. Although it is not clear how this is brought about, it is interpretable if on a nitrogen-free diet the chief deficiency is of methionine. The animal appears to break down proteins and waste other amino acids in order to obtain sufficient methionine. This is consistent with the fact that methionine has functions other than protein synthesis.

It is our conclusion from the present work that the common practice of depleting animals to low nitrogen excretion levels prior to studies on nitrogen requirements is not justified. Levels of nitrogen which apparently maintain such animals in nitrogen balance and also maintain plasma proteins and weight within the low normal range may not be adequate, as evidenced by liver pathology and failure of appetite. However, this level of nitrogen may still be adequate to maintain an animal which has not been depleted, especially if methionine is supplied. Studies similar to these but without prior depletion are needed for the answer to this problem.

## SUMMARY

In determining nitrogen requirements, it is recognized that some nitrogen depletion is necessary if minimum requirements are to be determined. Various investigators have considered it desirable to deplete animals to a minimum nitrogen excretion if minimum nitrogen requirements are to be determined. In this study 2 mg of nitrogen (as egg protein) per basal calorie were fed to dogs which were only partially depleted as evidenced by urinary nitrogen levels. Nitrogen balance, plasma protein, plasma albumin and hemoglobin concentrations, plasma volume, thiocyanate space, total circulating plasma protein, total circulating hemoglobin, body weight, liver analyses and microscopic examination at autopsy were used to evaluate the adequacy of the level of protein fed.

A slight fall in plasma protein and hemoglobin (both as per cent and as total circulating) was observed before minimum nitrogen excretion was reached. After this partial depletion all of the criteria except the last two in the foregoing list indicated that the animals were being maintained in this condition by the level of protein fed. Chemical analyses of the liver indicated considerable but not excessive nitrogen depletion. However, the microscopic examination of the livers revealed swelling of the liver cells, marked rarefaction of the cytoplasm, and loss of basophilic granules (ribonucleic acid). These changes are considered indicative of severe nitrogen depletion. Appetite also failed in most of the animals.

It is therefore concluded that (1) this level of nitrogen could maintain partially depleted animals insofar as the usual tests for nitrogen metabolism (nitrogen balance, plasma protein concentration) are concerned (2) the animals were too severely depleted to be considered normal as evidenced by the pathologic changes in the liver and the failure of appetite (3) the chemical and physiologic tests used are either inappropriate or insufficiently sensitive to determine the degree of nitrogen depletion indicated by liver examination and (4) further work using nondepleted animals is required to determine if dietary nitrogen fed at the endogenous level of nitrogen excretion will maintain normal animals.

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# A METHOD FOR THE DETERMINATION OF PLASMA CATALASE AND THE VALUES OBTAINED IN NORMAL ADULTS

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LOEW,<sup>1</sup> in 1901, demonstrated that there was a characteristic enzyme in all plant and animal cells with the specific action of decomposing hydrogen peroxide into water and oxygen which he named "catalase." In mammals, the erythrocytes liver and renal cells contain relatively much more catalase than do other tissues. The clinical importance of the catalase activity of whole blood has been shown to be little or none and the changes in health and disease have been shown to parallel the erythrocyte counts, although in general there has seemed to be less of this enzyme in the red cells in the various anemic states.

The catalase content of plasma or serum in human beings has received scant study. Becht<sup>2</sup> using relatively crude methods, found evidence of slight catalase activity in serum, as did Jolles and Oppenheim.<sup>3</sup> Oppenheimer<sup>4</sup> stated that the low values found in serum probably were the result of red cell destruction but he did not make clear whether he meant *in vitro* or *in vivo* lysis. Perlmann and Lipmann<sup>5</sup> showed that catalase is found in the various fractions when the proteins of serum or plasma are fractionated.

Description of a clinical method for the determination of catalase activity in serum or plasma has not been found in the literature. Kuokawa<sup>6</sup> presented observations on dog plasma based upon the original method of Jolles. He stated that, in the dog, bleeding had little effect on the plasma catalase activity, but that injection of distilled water, direct hepatic trauma, and hepatic poisons gave increased values.

We have developed, for the determination of catalase in plasma, a relatively simple clinical method which yields results that are reproducible within 5 per cent, most determinations checking within 2 per cent. This method is similar to that of Jolles in principle but it has been modified to increase the sensitivity for the small amounts of catalase which are found in plasma. The procedure is based upon the determination of the amount of hydrogen peroxide decomposed in a given time by a specific dilution of plasma under constant conditions. When a 1:50 dilution of plasma, in a solution containing 0.02N hydrogen peroxide and 0.8 per cent sodium chloride at a pH of 6.8 (0.006M phosphate buffer), is incubated at a temperature of 22 to 23° C. for twenty minutes, the decomposition of the peroxide in each cubic centimeter of the mixture is designated 1 unit of catalase activity. Hydrogen peroxide is measured by the determination of the amount of iodine liberated from iodide (catalyzed by molybdate), conventional thiosulfate titration being used.

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## MATERIALS AND SOLUTIONS

1 Powdered heparin The product of the Connaught Laboratory which contains 110 cat units per milligram is used This should be finely powdered and tested for catalase activity We have found no indications of the presence of catalase in the several lots used

2 Concentrated phosphate buffer and saline A 0.08M phosphate buffer at a pH of 6.8 is prepared and enough sodium chloride added to make an 8 per cent saline mixture This is sterilized by boiling and kept under sterile conditions to prevent mold growth which may have catalase activity

## 3 Superoxol

4 A 0.01N hydrogen peroxide buffer and saline solution This is made by adding about 0.6 cc of superoxol to 50 cc of the concentrated buffer saline mixture and diluting to 500 cubic centimeters Ten cubic centimeters of the resulting mixture should require from 0 to 2 cc of 0.01N thiosulfate This solution is made fresh daily and is used within four hours of preparation

5 A 0.01N thiosulfate solution This is made by diluting a 0.1N stock solution of sodium thiosulfate

6 A 10 per cent solution of potassium iodide

7 A 1 per cent solution of ammonium molybdate

8 A 5 per cent by volume sulfuric acid solution

9 A 1 per cent solution of starch

## 1 PROCEDURE

As much finely powdered heparin as can be obtained on the tip of a tooth pick is dusted into a 20 cc syringe A like amount of heparin is dusted into a 15 cc centrifuge tube which has a paraffin covered cork With a 19 gauge needle about 17 cc of blood are slowly withdrawn from an arm vein in the usual manner care being taken that bubbles of air are not sucked through the blood The needle is removed from the syringe and the blood is allowed to flow gently down the slanting side of the centrifuge tube until the tube is almost full The cork is inserted and the tube is gently inverted once The blood is then centrifuged at a moderate speed for fifteen minutes About half of the plasma is carefully removed and recentrifuged for a like period Then 0.2 cc of plasma in a Kahn pipette is added to 10 cc of the hydrogen peroxide buffer mixture and the solution is surged back and forth in the pipette to insure complete removal of all of the plasma A 120 cc Erlenmeyer flask should be used for this mixture The solution should be at a room temperature of 22 to 23° C if the temperature is above or below this a water bath kept at this temperature must be employed Twenty minutes later the enzymatic action is stopped by adding 10 cc of sulfuric acid solution A blank is run in an identical manner except that the sulfuric acid solution is added to the peroxide before the plasma is added Five cubic centimeters of the 10 per cent iodide solution and 3 drops of the solution of ammonium molybdate are added to each flask After three minutes the liberated iodine is titrated with the thiosulfate solution in the usual manner The volume, in cubic centimeters, of 0.01N thiosulfate solution of the blank minus that required by the plasma assay, multiplied by 25 gives the catalase activity of 1 cc of plasma in terms of the units previously defined If duplicates do not agree within 5 per cent, the test is repeated

Comment—It was found that the drawing of bubbles of air through the blood during withdrawal gives high values owing to destruction of erythrocytes

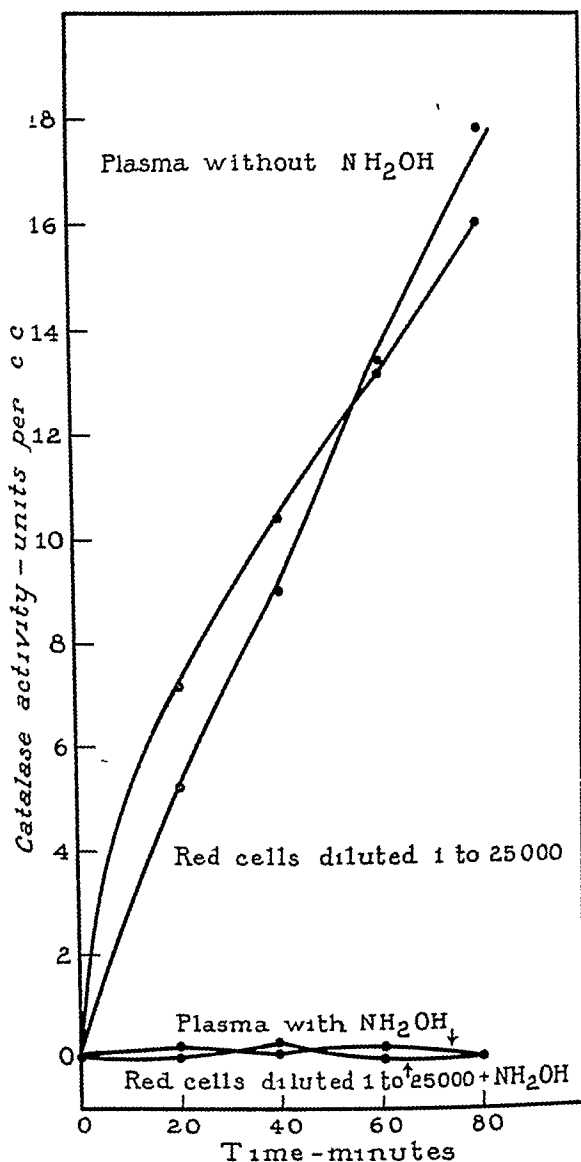


Fig 1—The effectiveness of hydroxylamine in inhibiting the catalase activity of lysed erythrocytes and plasma

For the same reason it is necessary to allow the blood to flow gently into the centrifuge tube and to avoid squirting. By comparison, it was found that heparin-treated plasma gives lower catalase values than does plasma prepared by the various citrate or oxalate methods. Serum also was found to give much higher values owing to cell destruction during coagulation. Cell-free plasma was found to lose its catalase activity upon standing, in one hour about 10 per cent loss occurs.

That the decomposition of hydrogen peroxide in the procedure just described is due to catalase in the plasma and not to the oxidation, by the peroxide, of the



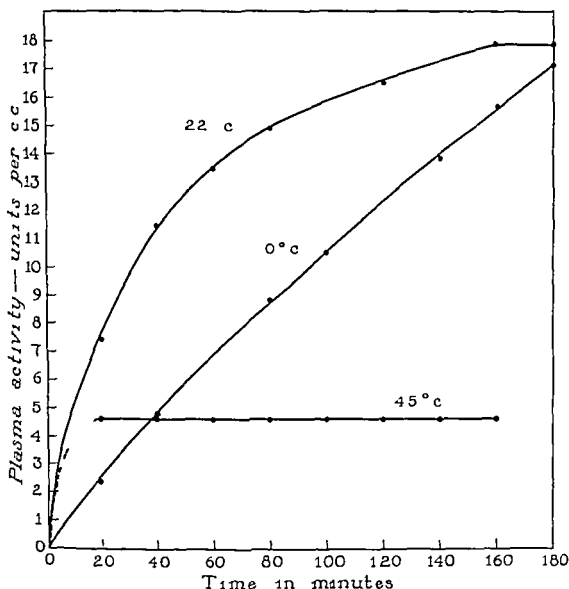


Fig 2—The effect of temperature changes on the catalase activity of plasma

many oxidizable substances known to exist in plasma was shown by various methods. Urine diluted 1:50, with enough glucose added to make a concentration comparable to that of plasma, gives no decomposition of peroxide under the conditions of the present method. It is well known that hydroxylamine is an inhibitor of the action of catalase. As shown in Fig 1 the presence of hydroxylamine completely inhibited the decomposition of hydrogen peroxide by plasma or by lysed erythrocytes.

Another proof that catalase activity is responsible for the observed action is the reduction of the maximal amount of decomposition of peroxide with temperature increases. When time and the amount of peroxide conversion are plotted at various temperatures, curves are obtained as in Fig 2. If non-enzymatic oxidation reactions were involved one would expect increased activity at higher temperatures, a plateau being reached only when all peroxide was consumed or all oxidizable substances were oxidized. It will be noted that at 45°C a plateau is quickly reached but the curves at lower temperatures indicate that this could not be due to lack of available peroxide or oxidizable substances. Likewise, the curve at 22°C shows a maximum of activity which is lower than the maximum reached at 0°C. The curves are explained by the known peculiarities of catalase. Unlike most enzymes catalase is rather rapidly inactivated by its substrate. This inactivation is at a minimum at freezing but rapidly increases with relatively small increments in temperature.

The question arises as to whether peroxidase activity may not complicate the estimation of catalase activity. This complication is unimportant, according to the following calculation. If one uses the liberal value for phenols, including tyrosine, epinephrine and bilirubin, in the blood plasma of 10 mg per 100 cc and, using the molecular weight of tyrosine, assumes that one mole of oxygen is used per mole of substrate, it can be calculated that not more than 0.008 mg of oxygen would be consumed per cubic centimeter of plasma, whereas the oxygen liberated from the hydrogen peroxide by 1 cc of plasma, the mean value for the method being used, amounts to 1.12 mg, hence, less than 1 per cent of the observed value could be ascribed to peroxidase action.

Several other factors indicate that peroxidase activity does not occur under the conditions of the test. No darkening of the reaction mixture, which one might expect if there had been any appreciable oxidation of phenolic compounds, was observed during the test. When plasmas containing much bilirubin—so much that the reaction mixture was a faint yellow—were tested, there was observed no change to the green tint during the enzymatic period which would have been expected if peroxidase activity had been present. It may be remarked that the bilirubin in these instances was oxidized to biliverdin, which gave a green tint, due to oxidation by the free iodine liberated during the assay of the hydrogen peroxide.

It would be difficult to prove that the catalase activity observed was not due to the destruction of erythrocytes during the collection and preparation of the plasma. It can be stated that the method presented gives the lowest values for catalase activity when compared with other methods of preparation of plasma or serum. As will be shown later in another paper on hemolytic diseases, in conditions in which an increase in plasma catalase would be expected, the values for catalase activity are actually much higher than the normal upper limit but, after correction of the hemolyzing process, the values become more nearly normal. That heparin itself is without effect on the erythrocytes is indicated by the fact that a specimen of blood containing heparin in the amounts specified in this method and another containing about ten times this amount yielded the same results in duplicate.

The use of paraffin-lined tubes and oiled syringe gave, in some instances, higher values when compared with heparin duplicates, in other cases closely checking results were found, but in no instance was a value lower than that of the heparin duplicate observed. The possible disruption of erythrocytes during centrifugation was minimized by the use of moderate speeds and by avoidance of rapid increases to top speeds.

A second problem presented itself, namely, whether the catalase activity of the erythrocytes influences the activity as observed in the plasma. This was investigated by making a 1:500 dilution of the erythrocyte layer after centrifuging, a 0.2 per cent solution of sodium carbonate being used as diluent. Then 0.2 cc of this lysed erythrocyte solution was assayed for catalase activity by use of the same method as for plasma except that a thirty-minute digestion period was used. The hemoglobin of the solution was determined by means of

a Photometer. The amount of hemoglobin expressed in grams per 100 cc of erythrocyte layer and divided by the catalase activity of 1 cc of the 1:500 solution of lysed erythrocytes was designated the hemoglobin catalase coefficient.

### RESULTS

The plasma of fifty adults was assayed for catalase activity by the method described. These people were all classed as normal inasmuch as the value for hemoglobin was more than 13.0 Gm per 100 cc of whole blood and they were considered free of serious disease or disorders in which one would anticipate tissue or blood destruction.

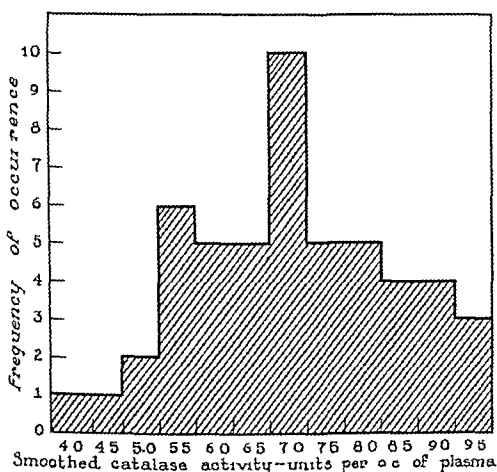


Fig 3—Distribution of catalase activity of the plasma in fifty one normal adults

Normal values were found to range from 42 to 95 catalase units per cubic centimeter of plasma, the mean was 69 catalase units. When these values were smoothed to the nearest whole or half number a distribution curve was obtained as shown in Fig 3.

The hemoglobin catalase coefficient varied from 2.4 to 3.3 in the twenty five cases in which it was determined. No correlation was found between this coefficient and the catalase activity of the corresponding plasma, showing that the catalase activity of erythrocytes does not influence the plasma activity.

The daily values for plasma catalase activity in a normal adult over a period of five days were 95, 85, 95, 70, and 95, respectively. Although but little fluctuation was found in this instance more determinations on various people are needed before definite conclusions can be drawn.

## SUMMARY

A method for the determination of catalase activity of human plasma has been presented. The normal values obtained in fifty adults were found to range from 4.2 to 9.5 catalase units per cubic centimeter of plasma, the mean being 6.9 catalase units. No correlation was apparent between the catalase activity of the erythrocytes and the catalase activity of the corresponding plasma.

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## PLASMA CATALASE IN HEMOLYTIC DISEASES AND OTHER ABNORMAL STATES

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A PREVIOUS report<sup>1</sup> has described a clinical method for the determination of plasma catalase and has established normal values for adults. By the use of this method abnormal states have been studied. The procedure has been varied slightly in that with the higher catalase values reached in disease states (with values greater than 50 units, which is the limiting value in the method as described) smaller dilutions of plasma were necessary. Usually 0.2 cc. of plasma in 50 cc. of the hydrogen peroxide and buffer mixture was used when there was reason to expect very high catalase values or when values of more than 50 were found using the regular procedure. The catalase coefficient of the erythrocyte layer was determined as described in the preceding paper.

Because erythrocytes, liver cells, and renal parenchyma are relatively rich in catalase, one might theoretically predict some of the known clinical conditions in which an increase in plasma catalase could be found. Any condition in which destruction of erythrocytes was occurring to a greater degree than normally occurs in the blood stream would be expected to give a rise of concentration of catalase in the plasma. Those states in which this might be anticipated include transfusion reactions, congenital and acquired hemolytic anemia, pernicious anemia in severe relapse, acute infections causing rapid anemia, paroxysmal hemoglobinuria, nocturnal hemoglobinuria, and the condition produced by injection of distilled water or other hypotonic solutions. Certain other conditions in which excessive destruction of blood is occurring with release of the contents of the erythrocytes into the blood stream include phlebotrombosis, infarctions of all types (especially myocardial infarctions with formation of large mural thrombi), hemorrhage into the thoracic or peritoneal cavities, and crushing injuries.

Conditions in which there is rapid destruction of hepatic parenchyma, such as acute yellow atrophy, acute hepatitis (infectious), poisoning from the various hepatic toxins, and crushing injury to the liver, would be expected to show some increase in plasma catalase.

There are few renal diseases in which rapid destruction of parenchyma usually occurs, but it is possible that poisoning by the heavy metals might show some change in plasma catalase activity.

We have investigated the plasma catalase in some of the disease states mentioned. Kurokawa<sup>2</sup> had found that in dogs injections of distilled water, trauma to the liver, and hepatic poisons gave increases in plasma catalase. We were

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TABLE I VALUES BEFORE, DURING, AND AFTER SPLENECTOMY IN FAMILIAL HEMOLYTIC ANEMIA

CASE	DAYS AFTER OPERATION	HEMO- GLOBIN (GM / 100 CC OF BLOOD)	ERYTHRO- CYTES (MIL- LIONS)	PLATIQ- UOUS CYTES (%)	SYSTEMIC CIRCULATORY BLOOD				SYSTEMIC VITROUS BLOOD				SYSTEMIC CIRCULATORY BLOOD				10*					
					PLASMA CATALASE (UNITS/ CC)	BILIRUBIN (MG /100 CC OF SERUM)		PLASMA CATALASE (UNITS/ CC)	BILIRUBIN (MG /100 CC OF SERUM)		PLASMA CATALASE (UNITS/ CC)	BILIRUBIN (MG /100 CC OF SERUM)										
						DI- RECT	INDI- RECT		DI- RECT	INDI- RECT		DI- RECT	INDI- RECT	DI- RECT	INDI- RECT							
1	0	4.7	1.0		27.4	1.1	3.9	30+	1.04	1.14	23	0.5	0.8									
	5	7.0			20.0	0.0	0.6															
	16	8.7			15.0	0.0	0.6															
2	Preoperative	14.4	4.7	5.3	10.0	0.0	5.2	23.7	0.0	6.8	15.8	0.0	5.3									
0																						
8					9.7	0.0	0.3															
3	0	10.8	3.7	18	34.5	5.3	3.5	31.5	4.9	3.1	33	4.9	2.5									
	13	12.9	4.8	0.8	26.0	1.0	0.8															
	17				22.5	0.0	1.2															
	21				17.0																	
4	0	12.1	3.9	27	7.5	0.0	3.5	10	0.0	4.3	9.9	0.0	4.1									

\*Hb = Hemoglobin of erythrocyte layer (Gm per 100 cc)

CA = Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution

able to investigate plasma catalase in hemolytic anemia in pernicious anemia, in anemia associated with renal insufficiency after transurethral resection and in sundry miscellaneous conditions

Table I summarizes the results obtained in hemolytic anemia at the time of splenectomy and after the operation. It is of interest that in two cases marked differences were found in the catalase of the blood plasma from the splenic artery and veins, and in these cases noticeable differences were also found in the bilirubin of the serum. In the other two cases only minor differences in both catalase and bilirubin were present. The difference between the plasma catalase of the splenic and systemic veins was probably due to catalase pickup during passage through the liver. The one case in which no difference between splenic vein plasma and systemic vein plasma was found showed considerable direct bilirubin, indicative of hepatic parenchymal disease. This may be the reason for the similar values. As would be expected there was not much difference in plasma catalase between the splenic arterial blood and the systemic venous blood. These results are a good indication that the method used measures the plasma catalase present *in vivo* and that the catalase activity found is not due to the destruction of erythrocytes during the collection of the blood and the preparation of the plasma.

The blood plasma catalase activity of systemic venous blood in two cases was within normal limits (although in one case a value of 10 was greater than the highest value found in fifty normal adults). In both cases however the anemia was mild and it would seem that the rate of destruction in the spleen was not too great, the liver probably removed most of the catalase produced in the spleen.

The catalase content of the erythrocytes in these cases was essentially normal as determined by the hemoglobin catalase coefficient

TABLE II VALUES IN VARIOUS CASES OF HEMOLYTIC ANEMIA

CASE	HEMO GLOBIN (GM / 100 C C OF BLOOD)	ERYTHRO CYTES (MIL LIONS)	Hb CA	CATALASE (UNITS/ C C)	BILIRUBIN (MG /100 C C OF SERUM)		REMARKS
					DI RECT	INDI RECT	
5	6.8	15	32	365	13	17	Acquired hemolytic anemia retic ulocyte count 40 per cent
6			25	85	00	18	Congenital hemolytic anemia splenectomy 5 years previously
7	14.6	49	35	77	00	05	Congenital hemolytic anemia splenectomy 18 years previously
8	9.4	31		317	00	36	Acquired hemolytic anemia
9	30	10	30	372	07	19	Acquired hemolytic anemia in ad dition transfusion reactions fol lowing incompatible blood
10	11.9	40	30	116	00	18	Congenital hemolytic anemia of many years duration large firm spleen reticulocyte count 57 per cent
11	10.8	37	36	48	00	22	Congenital hemolytic anemia re ticulocyte count 86 per cent

$$\frac{\text{Hb}}{\text{C.A.}} = \frac{\text{Hemoglobin of erythrocyte layer (Gm per 100 c c)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:100 dilution}}$$

TABLE III VALUES BEFORE AND DURING TREATMENT OF PERNICIOUS ANEMIA

CASE	DAY OF TREATMENT	RETICULO CYTES (%)	HEMO GLOBIN (GM / 100 CC OF BLOOD)	ERYTH ROCYTES (MIL LIONS)	BILIRUBIN (MG / 100 CC OF SERUM)		PLASMA CATA LASE (UNITS/ CC)	Hb* CA	REMARKS
					DI RECT	INDI RECT			
12	2nd before	14	73	18	00	17	73	25	Treated with 5 units liver extract and 5 mg folic acid per day
	0	14			00	17	53	27	
	4	24.2		17	00	09	12.6		Maximal reticulocyte response on this day
	10	10.0		22	00	04	3.7	28	
	19	18		35			8.0		
	29		11.2	40					
13	0	0.9	6.8	16			47.6		Treated with 15 units of liver extract per day
	5	20.5		20					
	7			21.8			10.2		
	14	7.9		31			5.2		
14	0	0.6	4.7		0.0	1.34	36.7		Treated with 15 units of liver extract every day for 16 days
	2	1.7			0.0	1.91	45.2		
	5				0.0	1.87	32.7		
	6	9.5			0.0	1.7	17.1	3.8	
	8	18.0			0.0	1.7	7.8		Maximal reticulocyte response 20.6 per cent on 10th day of treatment
	12	10.6			0.0	1.2	5.2	3.6	
	15	6.6					4.8	3.4	
15	6th before	1.0	8.2		0.0	2.4	46.0		
	0	1.5	8.2		0.0	2.8	43.0	2.8	Treated with 45 units liver extract for first 3 days, then 15 units twice a week
	6	21.7	11.8		0.0	1.4	12.5	2.8	
	9	15.3					9.1		
	14	10.2					9.5		
	19	4.0	11.0				5.8		
16	0	0.4	6.4		0.0	1.6	49.7	2.0	Treated with varying amounts of liver extract and 5 mg folic acid per day, pneumonia developed on two occasions, 4th to 8th day of treatment and 18th to 22nd day of treatment
	6	28.4	7.3				21.2		
	11	8.6					11.7	2.1	
	15	5.6	10.8				8.1	2.5	
17	0	0.2	6.4	2.1			17.6	3.0	15 units liver on 1st day of treatment and 30 units on 7th day of treatment
	4	13.5					9.0	2.8	
	9	19.5	9.5	3.0			7.1		
18	0	1.4		1.9			21.3	2.1	45 units liver extract 1st and 2nd days of treatment, 15 units on 6th, and 10th days of treatment, regular retic and RBC not done
	7	4.2		3.2			10.3		
19	0	0.9	8.8	1.9			24.7		Treated with 0.5 unit liver extract and 5 mg folic acid per day for 25 days
	2	2.9	8.8	1.9			32.5	2.6	
	5	11.1	10.5	1.8			15.0		Maximal reticulocyte response
	7	7.6	10.9	2.6			13.0		
	13	4.6	10.5	3.0			10.0		
	18	0.4	11.5				9.1		
	0	0.3	6.0	2.06	0.0	1.76	43.0	2.7	Maximal reticulocyte response
	6	14.0	6.6		0.0	1.0	10.6	2.6	
20	10	3.8	7.7	1.75	0.0	0.6	5.4	2.4	



TABLE III—CONT'D

CASE	DAY OF TREATMENT	RETICULO CYTES (%)	HEMO GLOBIN (GM / 100 C C OF BLOOD)	ERYTH ROCYTES (MIL LIONS)	BILIRUBIN (MG / 100 C C OF SERUM)		PLASMA CATA LASE (UNITS/ C C)	Hb C A	REMARKS
					DI RECT	IND RECT			
91	0	22	63	103	00	24	189	31	15 units of liver extract for 3 consecutive days
	6	182	77	173	00	09	98	27	
92	0	06	105	21			138		1 unit liver extract and 1 mg folic acid per day for 30 days
	9	15		23			152	26	
	4	70		29			115	25	Maximal reticulocyte response
	9	39		29			144	2	
	11	13		29			145		
	15	11		35			127		
	21		136	39			115	27	
93	0	12	85	22	00	07	97		1 unit liver extract per day for 28 days
	5	52	82						Maximal reticulocyte response
	10	30		25			58		
	15	8	100	35			70		

$\frac{\text{Hb}}{\text{C A}} = \frac{\text{Hemoglobin of erythrocyte layer (Gm per 100 c c)}}{\text{Catalase activity of erythrocyte layer per cubic centimeter of 1:500 dilution}}$

The results in patients with hemolytic anemia not operated on or who had undergone splenectomy are presented in Table II. It will be noted that the patients with severe acquired anemia had very high catalase values. The patients with congenital anemias who had undergone splenectomy had normal values. The remaining two patients with congenital hemolytic anemia who were not operated on at this time had only mild anemia with relatively low reticulocyte counts. The plasma catalase activity of systemic venous plasma was a little greater than normal in one case and normal in another, thus also was noted in two of the patients with hemolytic anemia in Table I who underwent operation. One can probably assume that because in these cases the spleen is the organ of destruction and the liver removes catalase from the blood plasma normal or near normal values would be found in the congenital types of hemolytic anemia except during marked exacerbations or crises whereas in the acquired types of hemolytic anemia in which the site of blood destruction is more general possibly in the blood stream itself higher values will be found.

Again the catalase coefficients of the erythrocyte layer were essentially normal indicating no great change in the catalase content of the erythrocytes as compared with normal amounts.

Table III presents the catalase and other pertinent values obtained before and during the treatment of pernicious anemia. That an excessive destruction of erythrocytes occurs in severe pernicious anemia is well known not only by the increased indirect serum bilirubin value but also by the increased urobilin and urobilinogen output in the feces. In the days before liver therapy, the finding of excessive hemosiderin at necropsy in cases of pernicious anemia was often noted.

As shown in Table III all the patients who had pernicious anemia except one showed rather marked increases in the plasma catalase activity. It is also apparent that a prompt fall to normal values occurred coincidentally with the

reticulocyte response In one case (Case 22) in which the reticulocyte response was not marked, the values fluctuated consistently above normal Fig 1 also illustrates the responses obtained in a typical case

The most plausible explanation for the excessive destruction of erythrocytes in pernicious anemia has been that the poikilocytic and anisocytic cells, which are so numerous, withstand the trauma of circulation more poorly than normal cells The administration of liver seems to correct the production of these abnormally weak cells immediately, those already present are soon destroyed and the normal rate of destruction of erythrocytes with normal plasma catalase values is quickly established, as is indicated by the cases presented Although this has been shown previously, using serum bilirubin values during treatment as an index of destruction of erythrocytes, the use of plasma catalase values gives a more direct proof of these rapid changes during liver therapy

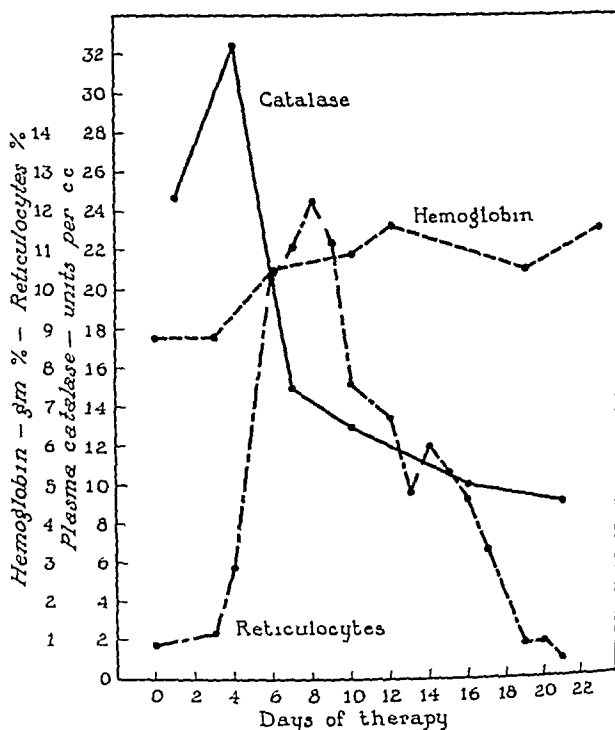


Fig 1—The effect of liver therapy on the plasma catalase hemoglobin and reticulocytes in pernicious anemia

The catalase coefficients are essentially normal and the changes during treatment do not vary enough to justify considering changes in the catalase content of the erythrocytes during treatment as a determining factor

The values for plasma catalase found in cases of anemia secondary to renal disease are given in Table IV The high catalase activity found in many of these cases was an unanticipated finding with no immediate explanation The bilirubin in these cases was not elevated nor has any previous evidence been found indicating that an excessive rate of destruction of erythrocytes was occurring in such cases of anemia Some patients were not anemic and none of

them were severely anemic. Because all of them were uremic it was possible that the abnormal amounts of metabolic end products in the plasma were being directly oxidized by the hydrogen peroxide, *but by using hydroxylamine it was shown that these values were due to catalase alone.* It is known that renal parenchymal cells are rich in catalase, but one can hardly explain these values by excessive destruction of renal tissue in the light of present knowledge of chronic renal disease. There was no correlation between the catalase activity in these cases and the blood urea, blood creatinine, or the inorganic constituents of the plasma.

TABLE IV VALUES IN ANEMIA SECONDARY TO RENAL DISEASE

CASE	HEMO GLOBIN (GM / 100 CC OF BLOOD)	ERYTHROCYTES (MIL LIONS)	PLASMA CAT ALASE (UNITS/ CC)	Hb* C.A.	UREA (MG / 100 CC OF BLOOD)	BILI RUBIN (MG / 100 CC OF SERUM)	REMARKS
4	8.6	3.0	More than 50	2.9	140	0.6	D & D + Group 4, with renal insufficiency and cardiac failure
5	1.6		38.7	3.0	220		Chronic glomerulonephritis patient died 4 days later
6	13.2	4.3	34.0	2.7	202		D & D, Group 4 or chronic glomerulonephritis differential diagnosis not possible
7	7.1	2.3	33.0	2.4	234	0.5	Chronic glomerulonephritis
8	8.4	3.9	18.0		146	0.3	Chronic glomerulonephritis
9	6.4	3.1	17.7		374	0.4	Chronic glomerulonephritis
30	1.4	4.0	16.5	2.3	78		D & D Group 3 possibly chronic glomerulonephritis pulmonary edema the previous night
31	10.1	3.9	16.0		156		Chronic glomerulonephritis possibly D & D with secondary renal insufficiency, death several weeks later
32	1.0	2.8	12.0	2.7	196	0.4	Chronic glomerulonephritis
33	7.8		11.7	3.0	134		Chronic glomerulonephritis
34	13.0		10.2	2.5	104		Kimmelstiel and Wilson's syndrome with diabetes mellitus
35	9.5	2.7	8.5		142		Chronic glomerulonephritis
36	13.1	3.9	8.5	3.0	128		Chronic glomerulonephritis
37	7.8	3.1	8.5	2.5	146		D & D with hypertension Group 4
38	10.6	2.8	7.9		178		Postsplenectomy Banti's disease, renal insufficiency cause unknown

Hb = Hemoglobin of erythrocyte layer (Gm per 100 cc)  
 C.A. = Catalase activity of erythrocyte layer per cubic centimeter of 1:400 dilution  
 \*Diffuse arterial disease

Again the catalase coefficients of the erythrocyte layer are within normal limits, a fact which shows that changes in catalase of erythrocytes were not a deciding factor.

It has been shown previously that hemolysis of blood occurs during prostatectomy.<sup>3</sup> This is probably due to the forcing of the distilled water which

is used to irrigate during the procedure into the venous openings in the prostatic beds. It was considered that the use of our method before and after prostatectomy would be of interest. Table V presents the results obtained. As indicated, in all but one case appreciable, and in many cases marked, increases of plasma catalase were found after prostatectomy. In several cases the plasma was grossly

TABLE V PLASMA CATALASE LEVELS BEFORE AND AFTER TRANSURETHRAL RESECTION IN WHICH DISTILLED WATER WAS USED AS AN IRRIGATION MEDIUM DURING THE PROCEDURE

CASE	POSTOPERATIVE TIME (HR.)	PLASMA CATALASE (UNITS/CC)	REMARKS
39	Preoperative	6.5	Plasma normal color
	3	24.0	
	24	11.5	
	48	11.0	
40	Preoperative	8.8	Plasma grossly red Plasma light pink Plasma normal color
	3	188.7	
	24	68.7	
	96	20.5	
41	Preoperative	4.0	
	1	15.8	
	3	16.5	
	6	10.5	
42	Preoperative	6.2	Plasma showed tinge of pink
	3	4.5	
	12	60.5	
	24	28.5	
43	Preoperative	12.6	Plasma grossly red Plasma light pink No apparent color
	3	9.5	
	12	174.0	
	24	93.5	
44	Preoperative	43.2	
	3	12.3	
	24	7.4	
	48	20.0	
45	Preoperative	14.5	
	3	10.2	
	12	9.5	
	3	19.0	
46	Preoperative	11.2	
	3	7.4	
	24	20.0	
	48	10.6	
47	Preoperative	9.0	
	3	13.2	
	24	7.7	
	48	11.2	
48	Preoperative	5.4	
	3	6.8	
	24	7.7	
	48	11.2	
49	Preoperative	5.4	
	3	6.8	
	24	7.7	
	48	11.2	

hemolytic. We consider these results an additional check on the validity of the method previously presented.<sup>1</sup> We also noted that these increased amounts of plasma catalase were not as rapidly removed as one would expect. Further studies comparing plasma catalase with plasma hemoglobin after prostatectomy would be of interest. We think that this method will be more sensitive than the present plasma hemoglobin procedures for detecting minimal hemolysis.

TABLE VI PLASMA CATALASE IN SOME MISCELLANEOUS CONDITIONS

CASE	HEMOGLOBIN (GM / 100 C.C. OF BLOOD)	ERYTHROCYTES (MIL LIONS)	BILIRUBIN (MG /100 C.C. OF SERUM)		PLASMA CATALASE (UNITS/ C.C.)	Hb* C.A.	DIAGNOSIS AND REMARKS
			DIRECT	INDIRECT			
50	13.5	5.1	11.6	4.4	65	25	Carcinoma of head of pancreas
51	10.3	3.24	8	1.1	61	3	Chronic hepatitis with a cites of 3 years duration
52	5.8	1.4			70		Aplastic anemia reticulocytes 0.3 per cent
53	14	4.5			41		Carcinoma of the stomach
54	10.3	4.2	0.5	0.2	78	25	Lymphoblastoma Hodgkin's type
55	10.5	4.9			72		Uremia secondary to prostatic obstruction
56	17.0	5.9	0.0	1.0	102		Polycythemia secondary to low vital capacity heart failure and residence at high altitude (5000 ft)
57	9.8	3.3			7		Chronic lymphatic leucemia
58	10.9	3.1			40		Carcinoma of prostate with metastasis
59	8.0	1.5	0.0	1.16	12.0		Cirrhosis of liver blood film showed 3 per cent reticulocytes macrocytosis increased regeneration
60	3.5	1.0		0.7	116	30	Chronic hypoplastic anemia some evidences of excessive destruction of erythrocytes (reticulocytes 78 per cent total urobilinogen 106 mg in 24 hour)
61	11.9	3.7	0.0	1.1	42.4		Diagnostic problem probably chronic mild hemolytic anemia required no spleen one episode of jaundice reticulocytes 31 per cent blood film showed increased regeneration and few spherocytes fragility 0.44 to 0.34 per cent
62	16.3	6.6			13.8	25	Polycythemia vera

Hb = Hemoglobin of erythrocyte layer (Gm per 100 c.c.)

C.A. = Catalase activity of erythrocyte layer per cubic centimeter of 1:100 dilution

Table VI gives values obtained in some miscellaneous cases. It is of interest that the presence of jaundice per se does not influence the values obtained. There is some indication that the high value found in Case 59 was due to increased destruction of erythrocytes (blood film and indirect bilirubin). Case 61 was of unusual interest. The patient had no splenomegaly or familial history. She presented a story of an attack of jaundice a year previously following sulfonamide therapy which was then considered to be responsible. The blood film increased indirect bilirubin, and increased catalase values would indicate that excessive destruction of erythrocytes was occurring. That in two cases of polycythemia the concentration of plasma catalase was greater than normal is of interest in that on theoretic grounds some increase would be expected. The catalase coefficient of the erythrocytes in the few cases in which this procedure was done again was within normal limits.

Because of the similarity of the chemical structure of catalase and hemoglobin, it is of additional significance that their ratio does not vary from normal in the various disease states studied

#### SUMMARY

A discussion of various disease states in which increased plasma catalase might theoretically be expected has been presented

Using a method previously described,<sup>1</sup> plasma catalase values have been determined in a number of disease states in which elevated levels might be expected. Elevated levels were found in acquired hemolytic anemias and in some familial hemolytic anemias. Differences in plasma catalase of splenic arterial blood and venous blood were found. Increased plasma catalase values were present in pernicious anemias and rapidly fell to normal with treatment. Increased amounts of catalase were present in the plasma in some cases of chronic renal disease. Moderate to marked plasma catalase activity was found after prostatectomy in which distilled water was used as the irrigating medium. Sundry other diseases in which excessive plasma catalase would not be expected gave normal values. The determination of plasma catalase may be of practical value in diagnostic problems.

The ratio of hemoglobin to catalase did not vary from normal in the disease states studied. Thus, variation in the catalase of erythrocytes was not a determining factor in the changes from normal found in plasma catalase in the cases presented.

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## EFFECT OF VARIOUS LETHAL PROCEDURES AND THERMAL INJURY ON CAPILLARIES

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THE role of capillary hyperemia in shock has long been a subject of controversy but in the past, errors of interpretation have arisen in part because of inadequate methods of tissue examination<sup>1, 2</sup> One potential source of error was uncovered when it was shown that congestion was often masked in unexsanguinated specimens<sup>3</sup> If an animal dies from shock and sufficient time is permitted before the organs are removed it is found that the organs of the shocked animals appear similar to or only slightly more congested than the corresponding organs of nonshocked animals On the other hand if the animals are killed by exsanguination the organs of shocked animals are much more congested than the organs of the unshocked animals Similarly when organs are removed in vivo, the difference in the amount of congestion in the shocked and unshocked animals becomes manifest only after the extirpated organs are permitted to bleed out

There is a second potential source of error which may invalidate many previous studies on congestion in shock The agent used to kill the experimental animal may in itself be responsible for a considerable amount of congestion In this instance fallacious conclusions would be drawn if the organs of an animal dead of shock were compared with the organs of a nonshocked animal destroyed by a lethal dose of anesthetic<sup>4</sup>

The method employed in this laboratory for estimating the degree of congestion is a comparison of the amount of hemoglobin and the number of capillaries in the exsanguinated organs of shocked and nonshocked animals The procedures for making hemoglobin assays<sup>5</sup> and capillary counts with the use of special stains for the erythrocytes<sup>6</sup> have been previously described

If these possible sources of error are taken into account it becomes evident why a pathologist who examines the tissues in the usual way is unable to determine with precision the amount of congestion in a given organ at autopsy In the first place he has no opportunity to estimate the amount of congestion by comparing exsanguinated organs Second he does not make hemoglobin assays and capillary counts Third, there is a possibility that terminal capillary atony occurs in death from a variety of causes other than shock

It is the latter possibility that requires further investigation, and to this end experiments were performed with a variety of lethal agents to determine to what extent they are capable of producing capillary hyperemia

*Experiment*—The object of the following experiment was to ascertain the amount of congestion which develops in a test visceral organ the kidney

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after a number of designated lethal procedures. A separate group of animals was subjected to fatal thermal trauma in order to compare the amount of congestion in a classic form of shock with that in the first group.

Forty-two Long-Evans rats weighing from 153 to 374 grams were divided into seven groups of six animals each and treated in the following manner. Under local anesthesia, with procaine, the right renal pedicle of each animal was tied and the kidney removed and allowed to bleed freely. The animals were then killed by various means. In the first five groups, death was produced by sodium pentobarbital (0.4 cc intravenously), procaine (0.4 cc intravenously), sodium cyanide (8 mg intravenously), ether (by inhalation), and asphyxia (by clamping the trachea), respectively. In the sixth group, death was caused by a severe burn (immersion to the thorax at 100° C for thirty seconds). In the seventh group, death was produced by rapid exsanguination from the abdominal aorta.

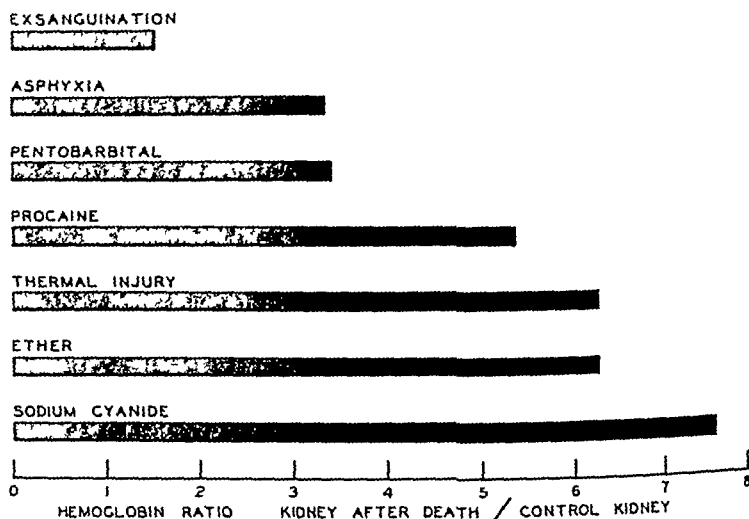


Fig 1—Relative amounts of hemoglobin retained in the exsanguinated kidneys of rats subjected to various lethal procedures. The control kidney was removed first under local anesthesia. After death the second kidney was removed and the hemoglobin content compared with the control. The pooled kidneys of six animals were used in each test.

The latter group served as a further control. Twenty minutes after death, in each instance the second kidney was removed and allowed to bleed out. The hemoglobin content of both kidneys of each animal was then ascertained.

**Results**—In every instance the second kidney contained a significantly greater amount of hemoglobin than the kidney removed before the animal was killed. In the seventh group, in which death was by exsanguination only, the increase was a small one. The findings are presented in Fig 1 in terms of the ratio of hemoglobin in the kidney removed after death to that in the control kidney.

This experiment shows that various lethal procedures as well as burns cause congestion in a test visceral organ and that the amount of congestion is quantitatively of the same order as that which develops in fatal burn shock.





A

B

Fig 2—Comparison of kidneys before and after a fatal burn. One kidney (A) was removed immediately before scalding of the body up to the head at 100° C for two minutes and is the control for the second kidney (B) which was removed three minutes after the burn. Both kidneys were exsanguinated after removal. Note the dark engorged appearance of the second kidney with little blood around it and the pool of blood around the pedicle of the first kidney after exsanguination.



A

B

Fig 3—Comparison of kidneys before and after a lethal dose of ether. One kidney (A) was removed immediately before administering ether and is the control for the second kidney (B) which was removed after fatal etherization. Both kidneys were allowed to self exsanguinate. Note the dark engorged appearance of the second kidney and the pool of blood around the pedicle of the first kidney.

This experiment confirms previous observations that the degree of congestion can best be demonstrated by making comparative examinations on exsanguinated organs.

In order to illustrate pictorially the effect upon the kidneys of a severe burn and a lethal dose of ether, respectively the following experiments were performed. Two animals were used. In each instance one kidney was removed

under light ether anesthesia and allowed to bleed out. The first animal was killed with ether, and the second by a severe burn. The second kidney of each animal was then removed and allowed to bleed out. Kodachromes were taken of the two sets of kidneys (Figs 2 and 3). The engorgement of the second kidney in each instance is striking.

#### DISCUSSION

If an animal dies from shock and sufficient time is permitted before the organs are removed, it is found that the organs of the shocked animal appear similar to, or only slightly more congested than, the organs of a nonshocked animal. In previous communications it was shown that the difference between the gross appearance of the organs of shocked and nonshocked animals becomes manifest if the animals are killed by exsanguination.<sup>3</sup> Obviously when tissues are taken for study some time after death has occurred, it is no longer possible to make such a comparison. The importance of exsanguination before examining the tissues in shock may explain previous failures to appreciate fully the vascular factor in shock.

In the present report it was shown that asphyxia, lethal doses of pentobarbital, procaine, sodium cyanide, and ether cause visceral congestion. In each instance the hemoglobin content of the kidney removed just prior to the administration of the fatal dose and allowed to bleed out was compared with that of the second kidney removed and treated similarly twenty minutes after death. When the two kidneys were similarly compared in the instance of death from exsanguination, it was found that the hemoglobin content of the second kidney was greater than the first, but that the amount of increase was much smaller than in the other experiments. The operative procedure involved in the removal of the first kidney causes a certain amount of trauma, and this could explain why in the exsanguination experiment the second kidney contained more blood than the first. In the experiments with the other lethal procedures, the difference in the hemoglobin content of the two kidneys was striking. Over seven times as much blood was found in the kidneys of the animals killed with cyanide.

These observations clearly show that capillary atony leading to visceral congestion develops from a wide variety of causes. The list includes burns, asphyxia, lethal doses of various drugs, and muscle crushing injury<sup>6</sup>; it is highly probable that death from many other causes is accompanied by capillary congestion. In determining whether visceral congestion is present in fatal shock, it would be a mistake to compare the degree of congestion in organs of animals in shock with that in corresponding organs of control animals sacrificed by a lethal dose of a substance like pentobarbital or ether. The utilization of these agents for the sacrifice of control, nonshocked animals may explain why many investigators have failed to find or have denied the existence of capillary congestion in shock. Mild or moderate anesthetic doses of these drugs do not cause a significant degree of visceral congestion,<sup>7</sup> but, as was demonstrated, lethal amounts do.

Since visceral congestion, if sufficient in degree leads to a reduction in the circulating blood volume or the venous return, the development of capillary congestion after lethal doses of certain chemical substances (pentobarbital, procaine, sodium cyanide and ether) and asphyxia suggests that the engorgement of the viscera may play an important role in the mechanism of death. As was demonstrated, the amount of congestion was fully as great in some instances as after thermal trauma. This raises the question of whether a shocklike process is not implicated in the mechanism of death after the administration of the chemicals investigated.

It may be possible to go a step further. Death from many causes may be accompanied by a significant degree of visceral congestion which the pathologist is unable to evaluate because of the factors mentioned. If the human heart is removed immediately after death from a large variety of causes it may with perfusion, be made to beat again, and this would indicate that the immediate cause of death was not failure of the heart. Is it not possible that in a wide variety of preagonal states due to infections, intoxications and so forth the sequestration of blood in atonic visceral capillaries may initiate a shocklike process which constitutes the final disorder of function which precedes and is the final cause of death?

#### SUMMARY

It was found that asphyxia and lethal doses of pentobarbital, ether, procaine, and sodium cyanide cause visceral hyperemia in a degree comparable in some instances to that which develops after fatal thermal trauma.

When a pathologist is called upon to decide whether or not hyperemia is present in the viscera of a shocked organism, he should bear in mind the results of comparative examination of organs after exsanguination and the fact that visceral congestion may be present after death from many causes other than classic shock, especially in organs of control animals sacrificed by lethal doses of anesthetic and other compounds.

It is suggested that the failure of some investigators to find unusual capillary hyperemia in shock is due to the visceral hyperemia which they have observed in control or nonshocked animals sacrificed by methods which in themselves cause intense visceral hyperemia.

We wish to extend our thanks to Dr Ben Sacks for valuable aid in the preparation of this manuscript.

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# THE MECHANISM OF BACTERIA-INDUCED SHOCK RESULTING FROM CRUSHING OF MUSCLE IN DOGS

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**I**N PREVIOUS studies, shock was induced in dogs by the crushing and replacement of surgically excised muscle. It was demonstrated that bacterial contamination of the traumatized muscle was responsible for the shock state, because, if bacterial growth was inhibited by suitable antibacterial agents, shock was prevented<sup>1</sup>. The amount of edema in the traumatized parts was too small to account for the development of shock.

In a series of experiments in rats and mice it has been shown that visceral congestion, due to atony of the vessels comprising the capillary bed, is a major factor in the initiation of the shock syndrome which develops after thermal trauma<sup>2</sup>. This factor operates by sequestration of blood in the dilated and atonic vessels of the visceral organs, which is demonstrable in exsanguinated animals or organs,<sup>2</sup> as a result of which the effective circulatory volume and the venous return fall to shock levels. In effect, the animal bleeds into its own capillary bed. In certain circumstances, local fluid loss is also a factor.

The purpose of the present investigation was to ascertain whether the factors operative in shock due to muscle crushing<sup>1</sup> are the same as those in thermal shock. Accordingly, experiments were conducted to determine whether the toxic factor from crushed injected muscle leads to capillary atony and a reduction in bleeding volume.

## METHODS

The quadriceps femoris of dogs anesthetized with sodium pentobarbital was removed from one hind limb. The muscle was cut into fine pieces, ground in a sterile mortar, and replaced in its original bed. The amount of muscle crushed corresponded to 3 to 5 Gm per kilogram of body weight. Sterile precautions were observed throughout. Experiment 1 As described in previous reports,<sup>1,3</sup> animals subjected to this procedure were obviously in shock at the end of twenty four hours. There was a decrease in the circulatory volume, the limb operated upon was edematous, and, despite the sterile technique, the crushed muscle at autopsy always had a foul odor, gas was often present, and direct smear of the muscle revealed numerous bacteria of many types.

**Bleeding Volume**—Under sodium pentobarbital anesthesia the femoral artery was cannulated and the blood received in a weighed container until exsanguination was complete. The bleeding volume is expressed as the weight of blood obtained as a per cent of the body weight.

**Hemoglobin Determination**—The amount of hemoglobin retained in two test organs, the kidney and the liver, after exsanguination of the animal was used as an index of the degree of capillary atony in the viscera. Hemoglobin was extracted from the finely cut organs with 20 per cent urea solution and readings were made on the Fisher Electro Hemometer. A more satisfactory method of determining hemoglobin in tissues has been developed subsequently and is described in detail in another publication.<sup>4</sup>

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*Histologic Examination*—The method of staining the tissues to facilitate the identification of the erythrocytes and the procedure for making capillary counts are described in a previous publication

*Experiment 1*—Under sodium pentobarbital anesthesia twelve dogs weighing from 7.8 to 17.2 kilograms were subjected to the crushed muscle procedure. Upon the development of profound shock (twenty to forty hours after the operation) each animal was reanesthetized and exsanguinated and the bleeding volume was determined. Sections of various organs (heart, liver, kidneys, spleen, adrenals, intestines and lungs) were taken for histologic study, and hemoglobin determinations were made of the liver and kidneys of each animal. For comparison, an equal number of normal animals was exsanguinated and investigated in the same manner.

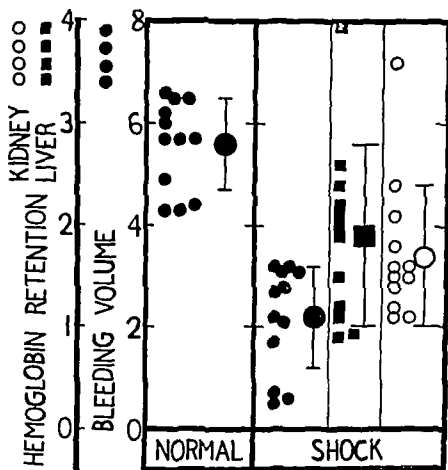


Fig 1—Graph showing relation of bleeding volume and hemoglobin retention in the liver and kidneys of dogs in shock produced by muscle crushing. Bleeding volume is expressed as the per cent of blood weight to body weight. Hemoglobin retention is in arbitrary units based on retention by normal tissue as unity. The large dots, square and circle represent the average of the values graphed. The vertical lines through these points are equal in length to 2 times the standard deviation and the horizontal lines above and below these represent the standard error of the mean.

In the shock experiments bleeding volume was significantly reduced and retention of blood in the liver and kidneys after exsanguination was significantly increased.

*Results*—The bleeding volumes of the shocked animals ranged from 0.53 to 3.2 per cent of body weight (mean 2.2 per cent) and those of the control normal animals from 4.3 to 6.6 per cent (mean 5.6 per cent) (Fig 1). These values for the bleeding volume in normal and shocked dogs are similar to those reported by other investigators.<sup>5</sup> The hemoglobin content of the kidneys and liver of shocked animals was considerably greater than that of the corresponding organs of normal control dogs (Fig 1). Histologic examination revealed that the number of open capillaries in the organs of shocked animals was increased.

and that the capillaries were wider and contained more erythrocytes than the corresponding organs of normal control animals. The examiner, without knowing which particular slide he was studying, was able in each instance to identify the organs of the shocked animals by the appearance of the capillaries.

In this experiment the physiologic changes which accompany shock, that is, visceral congestion and reduction in bleeding volume, are similar to those demonstrated in mice and rats after thermal trauma. In both instances a circulating chemical factor is responsible for the observed capillary atony. In the muscle-crushing experiment, this factor, a product of bacterial contamination, may be a bacterial toxin.<sup>8,9</sup>

*Experiment 2*—The following experiment was performed on four dogs in which each animal served as its own control. Under sodium pentobarbital anesthesia the left kidney of each animal was removed and the organ allowed to self-exsanguinate for twenty minutes. After exsanguination was complete, it was weighed, sections were taken for histologic study, and the hemoglobin content was determined. The muscle-crushing procedure was carried out immediately after nephrectomy. Approximately thirty hours later, while the animal was in profound shock, the opposite kidney was removed under sodium pentobarbital anesthesia and investigated in the same manner as the control kidney. The bleeding volume was measured immediately after the removal of the second kidney.

*Results* In each instance the kidney removed after the animal had entered into shock was more congested than the control kidney, it contained, on an average, more than twice as much blood as the normal kidney, and histologic study showed a large increase in the number of open capillaries, together with an increase in the diameter of and the amount of blood in every capillary. In three of the shocked animals the kidney weighed from 5.4 to 13.3 per cent more than the normal control kidney, and in the fourth animal the weights were the same, although the second kidney was more congested than the first. The bleeding volume in the shocked animals varied between 1.8 and 2.6 per cent of the body weight. This experiment, like the previous one, shows that a major factor causing the decreased bleeding volume in shock after muscle crushing is congestion of the visceral organs resulting from atony of the vessels comprising their capillary beds.

#### DISCUSSION

The results of Experiments 1 and 2 clearly indicate that shock due to muscle crushing results from a reduction in the effective circulatory volume and that a principal cause of this reduction is visceral congestion due to capillary atony. Thus, the shock-producing mechanism in crushed muscle injuries is similar to that observed in burns, except that in the former instance the toxic factor which leads to capillary atony is derived from infected muscle tissue. Shock induced by thermal injury in mice is due to a toxic factor not of bacterial origin because the shocked state may occur within a few minutes after severe injury.<sup>2</sup> Furthermore, the administration of antibacterial substances has no effect on the mortality after thermal injury.<sup>10,11</sup>

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# THE MECHANISM OF DELAYED DEATH FOLLOWING THERMAL TRAUMA

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IN PREVIOUS observations it was shown that burn shock was accompanied by a reduction in the circulatory blood volume, as represented by the bleeding volume, and that two major factors were implicated in this reduction—one, local fluid loss, and the other, atony of the vessels comprising the capillary bed of the visceral organs.<sup>1</sup> One of the methods employed for estimating the degree of capillary atony was measurement of the amount of hemoglobin retained in a test organ. In previous experiments it was shown that after a severe and fatal burn there is a progressive diminution in the bleeding volume, whereas after a less extensive burn there is an initial decrease followed by a gradual increase in bleeding volume as the animal recovers.<sup>1</sup>

In view of the fact that most of the previous observations were not carried beyond a twenty-four hour period,<sup>1</sup> it was considered of interest to ascertain whether any of the animals would succumb after apparent recovery from the initial period of shock. Observations showed that a number of animals died at various time intervals, from the second day to more than a month later, and an investigation was undertaken to determine the bleeding volume and degree of capillary atony in surviving animals in an endeavor to elucidate the mechanism of delayed death after thermal trauma.

## METHODS

The bleeding volume<sup>1</sup> was ascertained by cutting out the heart of the etherized animal and mopping up the blood entering the thoracic cavity with weighed cotton pledgets. The presence and degree of capillary atony were determined by measuring the amount of hemoglobin retained in the liver. Briefly, the method consists of extracting the hemoglobin from the finely cut liver with a buffered salt solution and measuring it photometrically after conversion to cyanmethemoglobin. The tissue hemoglobin values were corrected for polycythemia and anemia, respectively. Other details of the method have been described elsewhere.<sup>1, 2</sup>

All animals were placed upon a diet of Alber's Friskies, with the daily addition of 10 mg of ferrous sulfate, 0.2 mg of manganese chloride, and trace amounts of cobalt and copper salts per 100 ml of drinking water. These salts were added in an effort to counteract the anemia which was found to develop after the initial period of hemoconcentration in burned rats. In previous observations it had been shown that this diet with the salts added did not influence the bleeding volume.

*Experiment 1. Bleeding Volume and Hemoglobin Retention in Mice at Various Time Intervals After Thermal Injury*—Each of 412 adult male Swiss mice was anesthetized with ether and immersed up to the head in water at 60° C for seven seconds. The type of burn chosen was one which would permit most of the animals to survive for days or weeks after the initial period of shock. At intervals of two to eight hours during the first twenty-four hours, at the end of two and four days, respectively, and thereafter at stated intervals, a group of surviving mice were etherized and the bleeding volume and amount

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of hemoglobin retention in the liver ascertained. Upon each occasion when these determinations were made, control observations were taken upon a group of normal, unburned mice. The number of animals in the burned group which died each day was recorded and a mortality curve constructed (Fig 1).

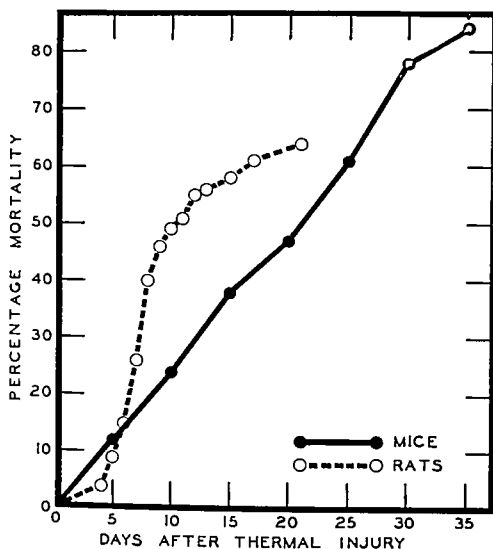


Fig 1—Time mortality curve based on (●) 300 mice scalded to the head in water at 60° C for seven seconds and (○) 80 rats scalded to the head in water at 65° C for ten seconds.

**Results** The changes in the bleeding volume and the amount of hemoglobin retention in the liver are shown in Figs 2 and 3. The greatest decline in the bleeding volume was noted in animals which were sacrificed four hours after the burn, at which time the value fell to an average of  $2.89 \pm 0.16^*$  per cent of body weight, as compared with the average control figure of  $5.86 \pm 0.10^*$ . It should be noted that the relatively mild type of burn employed in this experiment does not lead to the severest form of shock<sup>3</sup>. By the end of twenty-four hours the bleeding volume was back to almost normal. At the end of two hours the amount of hemoglobin retained in the liver was an average of 1.90 times that in the control, and at the end of four hours, 1.54. By the end of ten hours there was no longer an increased hemoglobin retention showing that the toxic vascular factor was no longer operative. In the subsequent observations, ending thirty-seven days after the experiment was begun, the figures for the bleeding volume returned to normal and there were no further increases above normal in the amount of the hemoglobin retention. Many of the values for hemoglobin retained in the liver fell below normal after the first day. Most of the animals became anemic and all the animals lost weight. A number which died during

\* Standard error of the mean

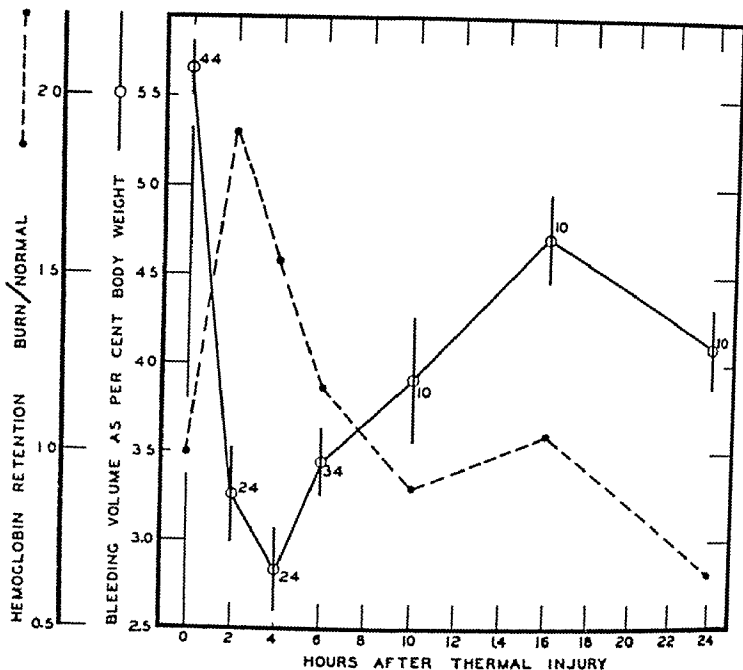


Fig 2—Relation of bleeding volume and hemoglobin retention to time after thermal injury (initial twenty-four hour period) Based on 100 mice which were immersed up to the head in water at 60° C for seven seconds. The verticle lines through the circles have a total length equal to three times the standard error of the mean. The numbers next to the circles represent the number of mice averaged for each point. For the hemoglobin retention graphs of this figure and Fig 3 the tissue hemoglobin of the burned animals was corrected by the ratio of the hematocrits of normal to burned animals. Within four hours after thermal injury the bleeding volume fell to a minimum value and the hemoglobin retained by the liver had already reached a maximum.

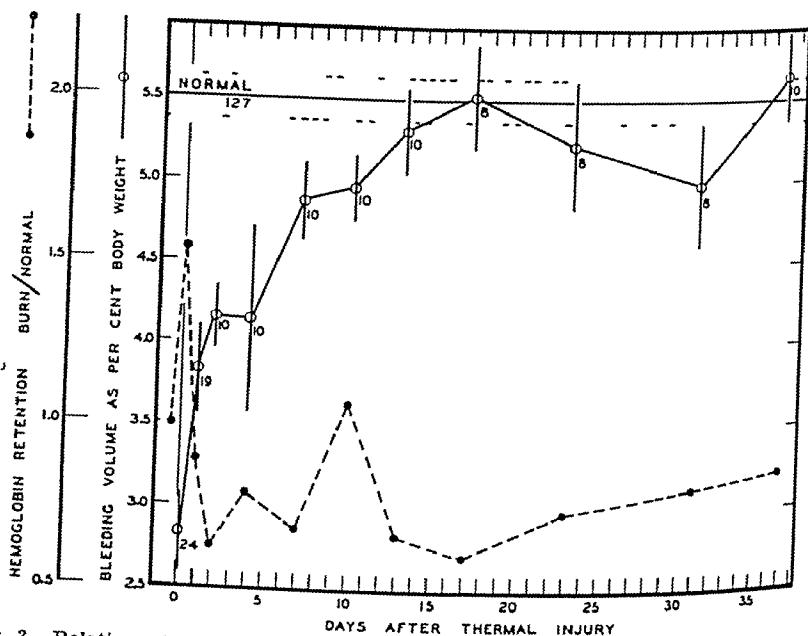


Fig 3—Relation of bleeding volume and hemoglobin retention to time after thermal injury (over a period of thirty-seven days) Based on 212 mice subjected to thermal injury. This is an extension of Fig 2 (the symbols are explained in the text of Fig 2). The dotted line accompanying the normal curve for the bleeding volume are separated by a distance equal to three times the standard error of the mean. During the thirty-seven days after thermal injury the bleeding volume returned to normal in about thirteen days although the amount of hemoglobin retained by the liver appeared to be somewhat less than normal in the burned mice.

the observation period showed infection or gangrene or both, in one or more limbs. The mode of death was in sharp contrast to that of animals which succumb to burn shock. In the latter instance, the skin temperature falls, dyspnea develops, and death occurs after a period of progressive asthenia and stupor. In the foregoing experiment, many of the animals appeared to be in good condition until very shortly before death and none died with the classic symptoms of shock.

*Experiment 2 Rate of Mortality and Bleeding Volume After Thermal Injury in Rats*—A group of 80 Long Evans rats, weighing from 250 to 400 grams, were anesthetized with ether and immersed in water at 65° C up to the head for ten seconds. This group together with an equal number of normal unburned rats was placed on the diet described under methods. The animals were observed for twenty six days. A mortality record was kept and a time mortality curve constructed. On the twenty sixth day all the surviving rats were anesthetized with ether and the bleeding volume was determined. Control observations were made upon an equal number of unburned animals.

*Results* In the rats which had survived for twenty six days the average bleeding volume was  $4.94 \pm 0.09^*$  in the controls. Statistical analysis shows that the amount of increase over the control is significant.

A few of the rats developed infection and gangrene of one or more limbs. As in the instance of the burned mice, a number of rats which succumbed at various time intervals after recovery from the initial period of shock appeared in good condition until shortly before death. The time mortality curve is given in Fig. 1.

#### DISCUSSION

These observations indicate that in mice which survive burn shock the bleeding volume returns to normal after twenty four hours, confirming previous studies with this type of burn.<sup>1</sup> Moreover, there was no increased retention of blood in the liver by the end of ten hours, suggesting that the toxic factor which causes atony of the vessels of the capillary bed was no longer operative. As the mortality curve (Fig. 1) shows, a certain number of mice died from two days to more than thirty days after the trauma. Surviving animals were selected at random for sacrifice on a particular day for determinations of the bleeding volume, but in order to ascertain whether the values in moribund animals differed from those in nonmoribund animals a number of the former were tested at the same time. The values did not differ significantly in the two groups. Neither decreased blood volume nor evidence of capillary atony was demonstrated in any of the animals which survived beyond the initial twenty four hour period.

Wilson and co-workers<sup>4</sup> followed the clinical course of treated burns in human beings and attributed the delayed deaths in their series to the presence of acute or septic toxemia, rather than to shock. The experiments reported in the present communication clearly show that delayed death in burned mice is not due to shock in the sense of a syndrome accompanied by decreased circulating blood volume and capillary atony.

Whereas the values for the bleeding volume returned to normal in the experiments on mice, the values were found to be above normal in rats which sur-

\* Standard error of the mean

vived twenty-six days after thermal trauma. Noble and Collip,<sup>5, 6</sup> who produced shock in rats by traumatizing them in a specially constructed drum, showed that animals which had been previously subjected to this form of trauma were able to withstand a second trial without being thrown into shock. These authors were unable to explain the immunity which had developed. If an increased blood volume were to be found in these animals, it would help to explain the immunity in the Noble-Collip experiments.

In the absence of visceral congestion and a decrease in the circulating volume, it is necessary to search for causes other than shock to explain the delayed mortality after thermal trauma. Surviving animals develop anemia and loss of weight and not infrequently infection or gangrene of the limbs. Whether the fatal outcome is a result of infection, a metabolic or nutritional disturbance, or is due to some other undetermined cause remains for future studies to explore.

#### SUMMARY AND CONCLUSIONS

The mechanism of delayed death in burned animals was investigated. The type of thermal trauma chosen for the experiment was one which produced shock but which enabled a large number of animals to survive for a number of days or weeks after the initial period of shock. Observations were made upon the circulatory blood volume, as represented by the bleeding volume, and on the degree of capillary atony, as represented by the amount of hemoglobin retained in the exsanguinated liver. These observations were begun two hours after the burn and continued for a period of thirty-seven days.

In mice, capillary atony and a decreased blood volume were demonstrated during the period in which the symptoms of shock were present. Upon the subsidence of these symptoms (generally within twenty-four hours), the blood volume was restored to its normal value, the capillary atony disappeared, and there was no further reduction in the blood volume or reappearance of visceral congestion throughout the period of observation.

In experiments on rats after similar thermal trauma, the bleeding volume showed a small but significant increase above the normal in observations made twenty-seven days after the burn. A possible explanation for the immunity to shock in the Noble-Collip experiment was suggested.

Delayed death after the type of thermal injury employed is not due to persistent or recurrent shock.

We wish to extend our thanks to Dr. Ben Sachs for valuable aid in the preparation of this manuscript.

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## LABORATORY METHODS

### A SIMPLIFIED SEDIMENTATION RATE TECHNIQUE WITH COMBINED CHART AND CORRECTION NOMOGRAM

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THIS paper describes a sedimentation chart and technique that permit correction for the effect of variations in red blood cell plasma ratio on the sedimentation rate. The method of correction by means of a nomogram printed directly on the sedimentation chart, is a simplified adaptation of that of Rourke and Ernstene.<sup>1</sup> The sedimentation chart (Fig. 1) is designed for inclusion in the patient's permanent record.

#### PRINCIPLE OF METHOD

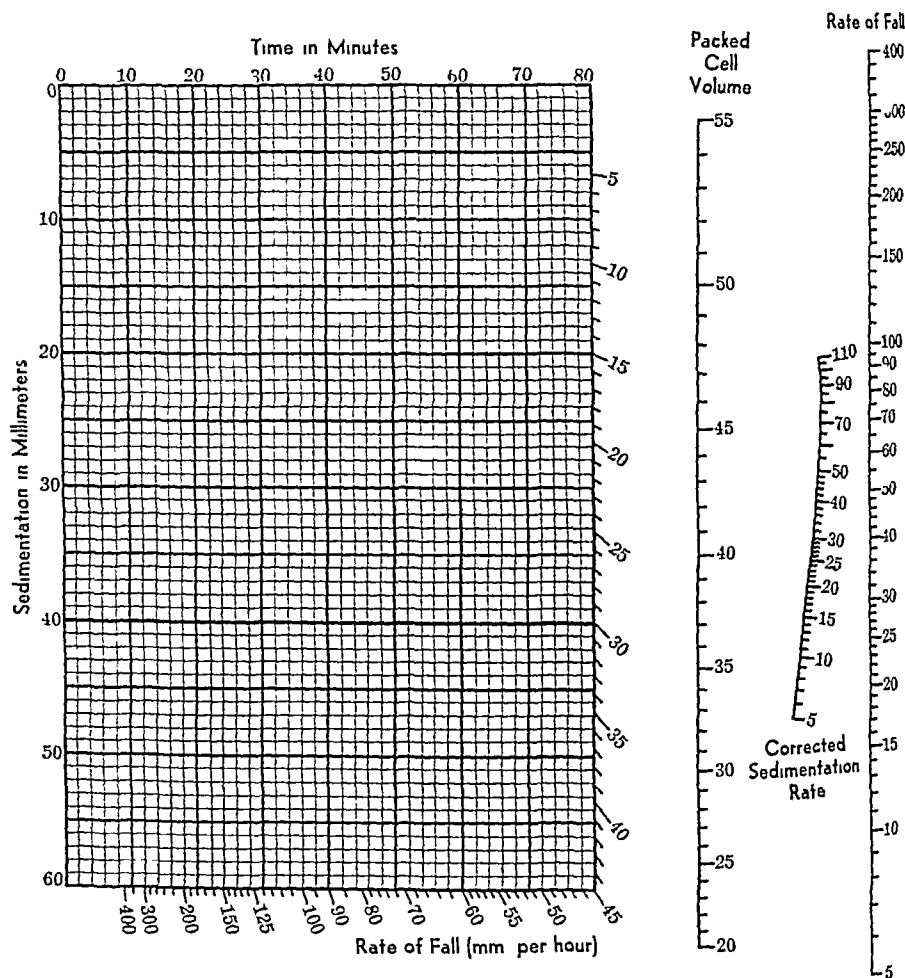
The sedimentation curve of uncoagulated blood obtained by plotting against time the settling of the topmost layer of cells in a vertical tube is typically sigmoid and may be divided into three components: (1) an initial slow phase or period of aggregation during which rouleau formation takes place and the rate of fall gradually increases; (2) a period of constant and maximum rate of fall, represented by a straight line; (3) a period of packing in which the rate becomes progressively slower as the cells pile up on the bottom of the tube. Sedimentation rate is sometimes defined as the total distance of settling in an arbitrary length of time, usually one hour. This almost always implies a summation of the first two or all three of the phases of sedimentation into a single value which may be the same for entirely different curves. The unambiguous definition of sedimentation rate as the maximum rate of fall, expressed by the slope of the second, straight line portion of the curve, has been adopted here.

It is possible to distinguish an elevation in sedimentation rate due to pathologic increase in certain constituents of the plasma, notably fibrinogen from that brought about simply by anemia. Such a differentiation is customarily made by expressing the sedimentation rate of a blood sample as that which would be observed at an arbitrary standard packed cell volume; the effect of variations in the cell plasma volume relationship is thereby eliminated. The two most frequently employed techniques involving a correction of this sort are those of Rourke and Ernstene<sup>1</sup> and Wintrobe and Landsberg.<sup>2</sup> In the latter method the number of millimeters of sedimentation in one hour is corrected for variations from a packed cell volume of 42 or 47 per cent; the average values for

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## SEDIMENTATION RATE REPORT

Name \_\_\_\_\_ Unit No \_\_\_\_\_ Clinic or Ward \_\_\_\_\_ Date \_\_\_\_\_



## OBSERVED DATA

Rate of fall (straight line portion of sedimentation curve) \_\_\_\_\_ mm. per hour

Volume of packed red cells \_\_\_\_\_ % of whole blood

## CORRECTED SEDIMENTATION RATE

(to packed cell volume of 45%) \_\_\_\_\_ mm per hour

(Description of method on other side)

## INTERPRETATION OF CORRECTED RATE

5-20 mm. per hour normal  
 20-45 mm. per hour slightly increased  
 45-75 mm. per hour moderately increased  
 Over 75 mm. per hour markedly increased

Fig 1—The report form

women and men, respectively. It has been shown that correction is more nearly accurate if based on the maximum rate of fall,<sup>3</sup> as in the method of Rouke and Einstene. In their technique, correction is made in the case of either sex to a packed cell volume of 45 per cent by means of a contour line chart constructed from experimental data. The method presented here is virtually the same, the only essential difference being in the simplified calculation of corrected rate through the use of a nomogram derived from the Rouke-Einstene chart.

## TECHNIQUE

For use as anticoagulant a solution is prepared of the double oxalate mixture of Heller and Paul<sup>4</sup> by dissolving 4.43 Gm of potassium oxalate and 6.87 Gm of ammonium oxalate (the monohydrate salt in each case) in distilled water to make a final volume of 500 cubic centimeters. This anticoagulant is cheaper and more stable than heparin and unlike the latter has no effect on the sedimentation rate.<sup>3</sup> One tenth cubic centimeter of the described 2 per cent solution corresponding to 2 mg of the salt mixture, is the optimum amount to prevent coagulation of 1 cc of blood. Small tubes or bottles are prepared by measuring into them and evaporating to dryness appropriate amounts of the solution (for example, 0.3 cc for 3 cc of blood). From 67 to 200 per cent of the designated optimum amount of blood may be used without clotting or alteration of packed cell volume or sedimentation rate<sup>3</sup> (namely 2 to 6 cc in the example given), this is a margin of safety useful in clinical work.

The appropriate measured amount of blood should be mixed with the anticoagulant as soon as possible after venipuncture.<sup>\*</sup> Within ninety minutes a chemically clean, dry sedimentation tube is filled to the 10 cm mark by means of a long stemmed capillary transfer pipette. The sedimentation in millimeters is accurately estimated at intervals of two, four, or six minutes depending on the speed of settling: the more rapid the sedimentation, the shorter is the period of constant fall, and hence the more frequently must observations be made.† Observations are begun approximately fifteen minutes after filling the sedimentation tube and continue until the trend of the plotted points shows that the third phase of the sedimentation curve has been reached. The tube is then centrifuged until packing is complete—about one half hour at 2,000 to 2,500 revolutions per minute—and the volume of packed red cells is read as percentage of whole blood.

After observations have been completed, a straight line is drawn through the points comprising the second phase of sedimentation. Another line is drawn parallel to the first, passing through the upper left hand corner of the chart (intersection of 0 coordinates) and extending to the scale on the right hand or lower margin, where the uncorrected rate of fall is read in millimeters per hour. Finally a straight line is drawn connecting points on the right and left hand scales of the nomogram, representing respectively the rate of fall and the packed cell volume. The point of intersection of this line with the middle scale indicates the sedimentation rate corrected to the standard packed cell volume of 45 per cent. The technique is illustrated in Fig 2.

<sup>\*</sup>One cubic centimeter of capillary blood is a satisfactory substitute for venous blood provided a sedimentation tube is used such as the Wintrobe which has a capacity of 0.7 cubic centimeter.

<sup>†</sup>In the presence of marked anemia not only is the rate of fall accelerated but in addition the top of the cellular layer is indistinct, due to the increased prominence of the effect of differential settling of erythrocytes in this zone. Precise individual readings are thus not possible but the rate of fall can be accurately estimated if many points are plotted.

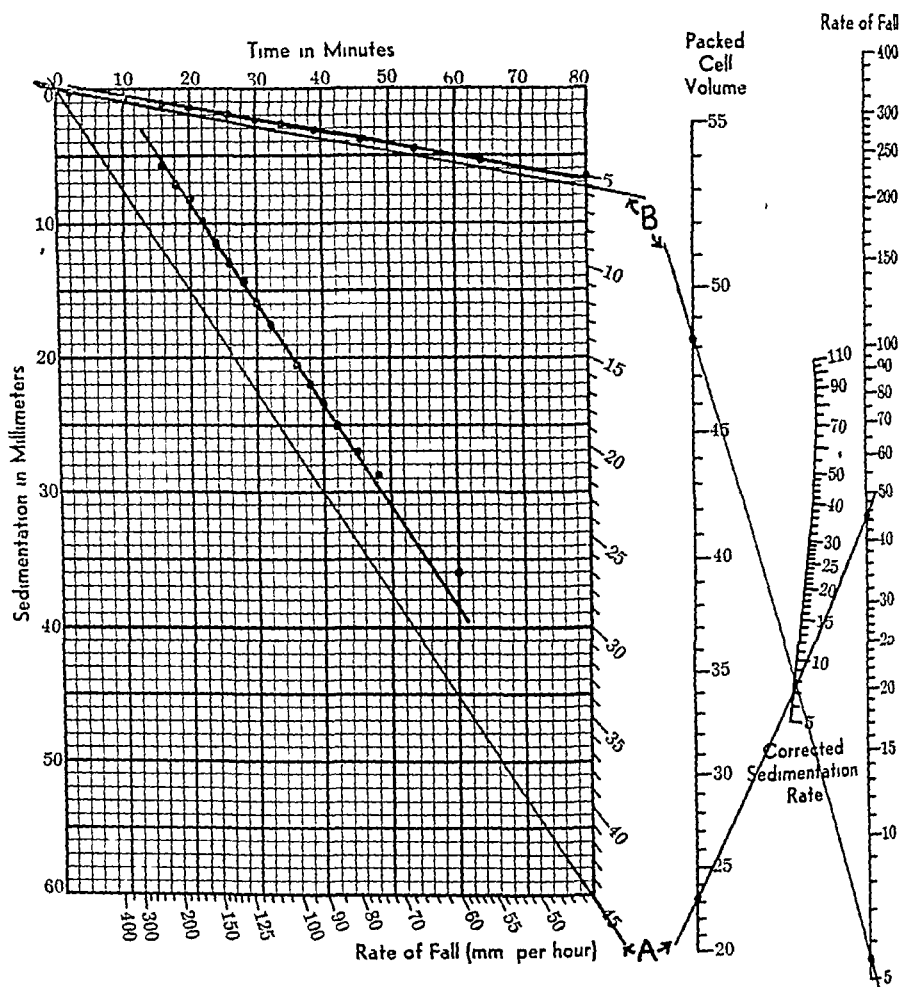


Fig 2—Effect of transfusion of washed erythrocytes on the sedimentation rate of a six month-old female infant with nutritional anemia A, Before transfusion B, two days later following transfusions

Up to twelve curves can be plotted concurrently by filling all the tubes and starting the observations approximately fifteen minutes after filling the first. Readings are plotted on a separate chart for each sample. A single observer needs only one stop watch or clock for the whole series of tubes, since they can be read in the same sequence each time.

The procedure is carried out at room temperature. The sedimentation tubes must be 3 mm or more in inside diameter<sup>3</sup> and are calibrated to contain a blood column 100 mm in height. Wintrobe<sup>5</sup> or Rourke-Einstene<sup>1</sup> hematocrits are satisfactory; a higher degree of accuracy in reading is possible with the former. It is important that the rack in which the tubes are set be stable and capable of being leveled so that the tubes are absolutely vertical. It should be placed at eye level to avoid errors of parallax. The tubes should be cleaned with acid



cleaning fluid frequently, preferably after each determination. After thorough rinsing with distilled water they should be allowed to dry by drainage and evaporation, rapid drying with alcohol and ether leaves a surface film which may produce retarded and irregular sedimentation.

#### SEDIMENTATION CHART AND NOMOGRAM

The sedimentation chart is represented in Fig 1\*. As printed it contains on the reverse side a brief description of the method. The construction of the nomogram was made possible by a transformation of coordinates so that the curved contour lines of the Rourke Ernstene chart could be plotted as a series of straight lines. The accuracy of the transformation is shown by the fact that values for corrected sedimentation rate obtained by the nomogram agree with those obtained by the Rourke Ernstene chart within 2 mm per hour except for packed cell volumes near 55 per cent where the deviation may be twice this amount. Values for corrected rates of less than 5 mm or more than 110 mm per hour, the limits of the scale, are rarely encountered.

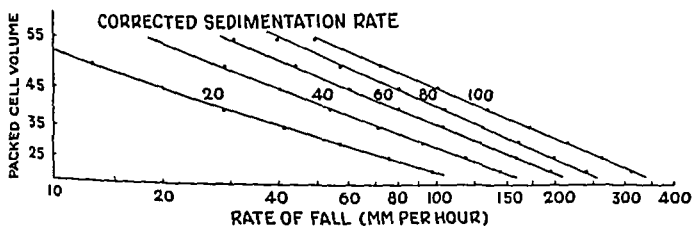


Fig 3—Transformation of coordinates of Rourke Ernstene chart. The straight lines represent corrected sedimentation rate in millimeters per hour.

#### DETAILS OF CONSTRUCTION OF NOMOGRAM

Simultaneous values for packed cell volume, rate of fall, and corrected sedimentation index were taken from the Rourke Ernstene chart and assembled in tabular form. The second and third variables were expressed in millimeters per hour instead of millimeters per minute. Corrected sedimentation index is hereinafter referred to as corrected sedimentation rate.

A method of graphic presentation was next sought that would permit plotting corrected sedimentation rate in terms of the other two variables in such a manner that the locus of the points representing any particular value for corrected sedimentation rate would be a straight line. By trial and error and inspection of the plotted points it was found that this could be accomplished most closely when  $\log (85 \text{ minus packed cell volume})$  was plotted against  $\log (\text{rate of fall})$ . The technique is illustrated in Fig 3 in which five

representative values for corrected sedimentation rate have been plotted in terms of packed cell volume and rate of fall. A series of straight lines was obtained for corrected sedimentation rates of 5 through 110 mm per hour. All lines were drawn by inspection.

From this series of straight lines, adjusted values for packed cell volume and rate of fall were obtained and tabulated. It is apparent from Fig 3 that the necessary adjustments were virtually negligible except in the case of many points corresponding to a packed cell volume of 55 per cent. The necessity for larger adjustments at this end of the scale explains the lack of uniformly close agreement between the nomogram and the Rouke-Einstene chart in this range, pointed out in the foregoing section.

The nomogram could then be constructed by placing the coordinates of Fig 3 parallel to each other instead of at right angles. The distance between the lines and the length of the scales were chosen arbitrarily. By this maneuver the straight lines representing corrected sedimentation rate became a series of points lying between the parallel scales. Each point in the series was located by the convergence of three straight lines drawn between widely separated positions on the outer scales representing simultaneous values for rate of fall and packed cell volume. Finally a smooth line was drawn by inspection through the series of points thus located.

The limits of the scale representing packed cell volume are 20 and 55, those of the Rouke-Einstene chart. The upper limit of the middle scale is 110, higher values for corrected sedimentation rate cannot be included since they are represented by curves rather than straight lines in the original transformation of coordinates (Fig 3). The lower limit of this scale corresponds to that of Rouke and Einstene. In the scale representing rate of fall, rates below 5 are not included because the nomogram would be made unwieldy, such rates are, in fact, infrequently encountered. Values above 360 represent an extrapolation since this value was the highest employed in the construction of the nomogram.

#### DISCUSSION

The sedimentation rate of ovalated blood is constant for a period of two and one-half hours following collection of the samples, thereafter there may be a decrease in rate, hence the need for beginning observations within ninety minutes. Zero time on the coordinate chart is quite arbitrary and for purposes of convenience will correspond in most instances to the time of the initial observation. Humps in the sedimentation curve may indicate a dirty tube or hemolyzed or partly clotted blood, the curve should be smooth and sigmoid and the points of the second phase should fall exactly on a straight line. The second phase ordinarily begins fifteen to thirty five minutes after the tube is filled and lasts fifteen to thirty minutes. It is thus necessary neither to start observations at once nor to continue them for a fixed period of time.

The precision of this technique is illustrated by the examples given in Figs 2 and 4. Fig 2 illustrates the effect of a variation in packed cell vol

ume on the observed sedimentation rate, while the corrected rate remains practically unchanged. In Fig. 4 is shown the correspondence of the corrected rates for venous and capillary blood in the same patient.

The corrected sedimentation rate of normal persons varies from 5 to 20 mm per hour, corresponding to a corrected sedimentation index of 0.08 to 0.35 in the Rourke-Ernstene method. Rates between 20 and 45 mm per hour are considered slightly elevated, between 45 and 75, moderately elevated and over 75 markedly elevated.

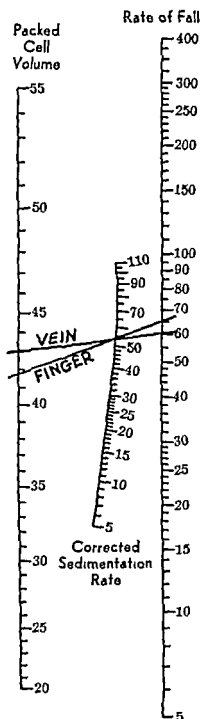


Fig. 4.—Sedimentation rates of capillary and venous blood samples simultaneously withdrawn.

This method has proved its practicability in seven years of use in the Hospital of the University of Pennsylvania.

#### SUMMARY

The determination of sedimentation rate according to the Rourke-Ernstene technique has been simplified through the use of a report form with nomogram, by means of which the observed rate may be corrected for variations

in packed cell volume from the standard value of 45 per cent. This correction permits a more accurate interpretation of sedimentation rate through the elimination of one extraneous effect, the varying red blood cell plasma ratio.

Dr. David Black proposed the inclusion of slopes on the margin of the coordinate chart to obviate the calculation of the rate of fall. We are indebted to him for this suggestion and for helpful advice in many other matters.

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# A DEVICE TO FACILITATE THE CLEANING OF CAGES CONTAINING COTTON RATS

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THE jumping ability of cotton rats is familiar to anyone who has handled these animals. If any appreciable number of cotton rats is in use, the cleaning of cages involves the expenditure of much time and energy.

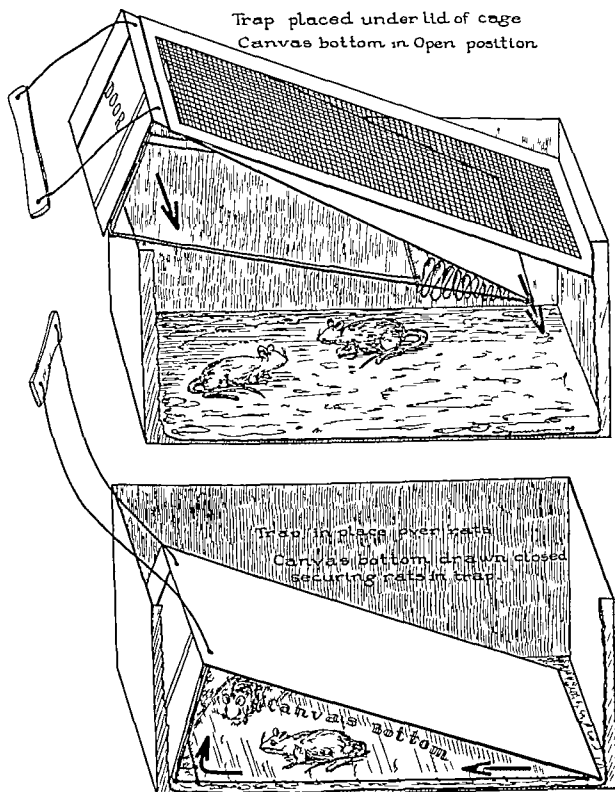


Fig 1

From the George Williams Hooper Foundation University of California Medical Center  
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The procedure formerly used in the animal quarters of the George Williams Hooper Foundation consisted of placing the cage in a large galvanized garbage can, removing the lid of the cage, placing the rats in the bottom of the can, and then cleaning the cage. After the cage had been cleaned, the rats were caught individually with forceps and placed in the cage. In addition to being time consuming, this procedure occasionally was responsible for rupture of the spleen or liver of the animals.

Hence, to facilitate cleaning the cages, the device shown diagrammatically in Fig. 1 was made. It is easily made with readily available materials. It is used with cages of the usual type, which have the lid on top. The device consists essentially of a metal box with tapered sides and with a simple sliding floor or stout canvas or flexible metal screen. No dimensions are given here, since it is necessary to adapt the size of the apparatus to the size of the cages in use.

In operation, the device is dropped into the cage by sliding it under the lid of the cage as the lid is raised. With the rats under the device, the lid of the cage is removed completely and the sliding floor is pulled into position. The device containing the rats, is then lifted from the cage. After the cage has been cleaned and fresh bedding and feed have been supplied, the rats are quickly and easily returned to it by means of the sliding door at the broad end of the device.

Under actual conditions of use, an experienced animal keeper required slightly less than one minute per cage to clean a total of 182 cages. Without the facilitating device, the same worker required about three minutes per cage.

## PENICILLIN IN THE TREATMENT OF ACTINOMYCOSIS

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IN 1943 Florey and Florey<sup>1</sup> reported the results of the treatment with penicillin of two patients who had actinomycosis. In these patients penicillin appeared to have no effect on the infection. However, in the light of our present knowledge the amounts of penicillin used and the method of administration employed appear to have been inadequate. In 1943 the Committee on Chemotherapeutic and Other Agents of the National Research Council<sup>2</sup> also reported data on the use of penicillin in three patients who had actinomycosis. One patient improved and two died. No details as to the type of case or the method or duration of treatment were given. In 1944, in collaboration with Heilman we<sup>3</sup> reported our initial observations on the use of penicillin in the treatment of twelve patients who had actinomycosis. No conclusions could be reached from these limited observations because the patients had been followed for inadequate periods. Treatment with penicillin of small groups of patients who had actinomycosis has been reported by other investigators.<sup>4-6</sup> The results have been encouraging.

Up to the present time, sixty patients who had actinomycosis have been treated with penicillin under our supervision. Fourteen of these patients have not been followed for a sufficient length of time to enable us to evaluate the results of treatment. Our present report, therefore, deals with our observations on the use of penicillin in the treatment of forty-six patients who had actinomycosis. These forty-six patients have been followed for periods of from one to five years since the conclusion of the treatment.

Considerable confusion and difference of opinion exist concerning the definition of the term actinomycosis. For the purposes of our investigative work we have confined our studies to infections caused by the microaerophilic organism *Actinomyces bovis*. Infections caused by the several species of the genus *Nocardia* have been classified separately and are not included in this report. The diagnosis of actinomycosis in each of our patients was made by direct examination or culture of pus obtained from a draining sinus or from material obtained at operation. All strains of *Actinomyces bovis* cultured were found to be sensitive to penicillin in vitro, the organism being inhibited by 0.01 to 0.1 unit of penicillin per cubic centimeter of culture medium.

In attempting to evaluate methods of treatment it is of importance to qualify carefully the general term actinomycosis, for the prognosis varies greatly according to the location of the lesion, the duration of the infection and the

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general condition of the patient. A method of treatment which is effective when the soft tissues of the face and neck are involved is often entirely ineffective when actinomycosis involves other parts of the body. If the disease has been present sufficiently long to permit an overgrowth of fibrous tissue or an impairment of the general health of the patient, effective treatment of any type will be much more difficult. A classification of actinomycosis according to the organ or organs involved is the most desirable. However, inasmuch as the pathologic process and the prognosis are similar when certain regions of the body are invaded, for purposes of discussion a more general classification can be adopted. Our patients, therefore, have been grouped under five general headings: cervicofacial actinomycosis, pulmonary actinomycosis, abdominal actinomycosis, pelvic actinomycosis, and actinomycosis involving other parts of the body.

Of the group of forty-six patients on whom we are reporting data, twenty-six were suffering from cervicofacial actinomycosis, nine from pulmonary actinomycosis, eight from abdominal actinomycosis, and three from pelvic actinomycosis. Penicillin was administered to each of these patients either by intermittent intramuscular injections every three hours or by the continuous intravenous drip method. The dosages of penicillin varied widely between 80,000 and 1,000,000 units daily. In most cases penicillin was administered continuously for periods ranging from two to seven weeks. In some instances the penicillin was administered in courses of ten days each, with intervals of one to several weeks intervening.

#### RESULTS ACCORDING TO LOCATION

*Cervicofacial Actinomycosis*.—Twenty-six patients with cervicofacial actinomycosis received penicillin. Since 1940, twenty-five other patients with cervicofacial actinomycosis have been seen by our colleagues or by ourselves. These patients did not receive penicillin but were treated by other methods. The number of patients who recovered was approximately equal in both of these groups, that is, in excess of 90 per cent (Table I). The significant difference between the two groups, however, appears to be the duration of treatment necessary to bring about recovery. When penicillin was used, satisfactory results were obtained after an average period of treatment of less than two months. When penicillin was not used, it was necessary to continue the treatment, on an average, for nearly six months. These figures include the entire period from the onset of treatment to the discontinuance of all forms of therapy. In both groups treatment was carried out continuously in some cases and at varying intervals in others.

TABLE I. PENICILLIN THERAPY FOR CERVICOFACIAL ACTINOMYCOSIS

TREATMENT	NUMBER OF CASES	RECOVERY		FAILURE		AVERAGE DURATION OF TREATMENT (MO.)
		NUMBER	PER CENT	NUMBER	PER CENT	
Penicillin	26	24	92	2	8	1.5
No penicillin	25	24	96	1	4	5.9



*Pulmonary Actinomycosis*—The most striking results of treatment with penicillin occurred in the patients who had pulmonary, abdominal, or pelvic actinomycosis, since patients with these types of actinomycosis had uniformly poor prognoses when other types of treatment were used. Nine patients who had pulmonary actinomycosis received penicillin (Table II). In this group there were five recoveries and four failures. Since 1940 thirteen other patients who had pulmonary actinomycosis have been seen by our colleagues or ourselves. None of these patients received penicillin but they were treated by other methods. Only one of them appears to have recovered.

TABLE II. PENICILLIN THERAPY FOR PULMONARY ACTINOMYCOSIS

TREATMENT	NUMBER OF CASES	RECOVERY		FAILURE	
		NUMBER	PER CENT	NUMBER	PER CENT
Penicillin	9	5	56	4	44
No penicillin	13	1	8	12	92

*Abdominal and Pelvic Actinomycosis*—Eight patients who had abdominal actinomycosis and three patients who had pelvic actinomycosis received penicillin (Table III). Six of those who had abdominal actinomycosis recovered and two did not. All of those who had pelvic actinomycosis recovered. Of sixteen patients with abdominal actinomycosis who did not receive penicillin only three appear to have completely recovered.

TABLE III. PENICILLIN THERAPY FOR ABDOMINAL AND PELVIC ACTINOMYCOSIS

TREATMENT	NUMBER OF CASES	RECOVERY		FAILURE	
		NUMBER	PER CENT	NUMBER	PER CENT
Penicillin	11	9	82	2	18
No penicillin	16	3	19	13	81

#### COMMENT

The results of this study appear to indicate that penicillin is an effective agent in the treatment of actinomycosis, its effectiveness varying somewhat with the location of the lesion. As is true in most infections, actinomycosis varies greatly in different individuals. Apparently in some cases of mild actinomycosis complete recovery from the infection occurs without treatment.<sup>10</sup> How often spontaneous recovery occurs is difficult to determine for it is not until the disease has become quite extensive that a clinical and bacteriologic diagnosis can be made. However, when the infection has become well established spontaneous cures occur only rarely.

Cervicofacial actinomycosis has responded well to several methods of treatment.<sup>11</sup> Prolonged surgical drainage, roentgen therapy, and administration of the iodides and some of the sulfonamide compounds have proved of value in the treatment of actinomycosis when it involves the neck or face. However, penicillin appears to have definitely shortened the duration of the infection and the period of treatment. In cases of cervicofacial actinomycosis of short duration

the use of penicillin alone has resulted in cures. When the infection has been extensive and of long duration, use of penicillin has been combined with surgical treatment. In most of the cases in which penicillin has been used, the sinuses closed rapidly, and the patients recovered completely in a relatively short time. Even the two patients who did not recover completely improved markedly while under treatment. Drainage from the sinuses ceased entirely, and the infection appeared to be cured at the time treatment was discontinued. However, in these two patients evidence of active infection appeared again several months later.

In some cases of cervicofacial actinomycosis death occurs from extension of the infection into the meninges. One of our patients was critically ill, with evidence of meningitis secondary to cervicofacial actinomycosis. Penicillin was administered for six weeks and the patient recovered.

Pulmonary actinomycosis nearly always has been a progressive and fatal disease. The prognosis has been particularly unfavorable when the parenchyma of the lung has been invaded. The usual methods of treatment have been ineffective in most cases. The recovery, after treatment with penicillin, of five of our nine patients who had pulmonary actinomycosis is, therefore, most encouraging. Moreover, two of the patients for whom the results are listed as failures are in good general condition, although they still have evidence of active disease.

Abdominal actinomycosis has always been a serious disease, but good results have been obtained in occasional patients by several different methods of treatment.<sup>12</sup> However, the percentage of patients who recovered has never been very great and the prognosis, therefore, has been poor. Seventy-five per cent of the patients with abdominal actinomycosis who received penicillin recovered. It appears, therefore, that penicillin is a very effective chemotherapeutic agent in the treatment of abdominal actinomycosis.

Actinomycosis involving the pelvic viscera in women may well be merely a localized form of abdominal actinomycosis. However, the prognosis when actinomycosis involves the pelvic organs has been extremely poor, and very few recoveries ever have been reported. The recovery, therefore, after treatment with penicillin, of the three women who had actinomycosis involving the pelvic viscera seems particularly significant.

In the treatment of all types of actinomycosis, a dosage of at least 500,000 units of penicillin daily administered intramuscularly or intravenously for a period of six weeks appears to achieve the best results. Adequate drainage is indicated if abscesses are present.

Because we have been attempting to evaluate the effectiveness of penicillin in the treatment of actinomycosis, other forms of therapy, aside from surgical drainage or excision of diseased tissue, have been avoided so far as possible in the treatment of our patients. It seems possible, therefore, that even better results can be achieved in the future if administration of adequate amounts of penicillin is combined with the use of sulfonamide compounds or of streptomycin.

## SUMMARY

Forty-six patients who had actinomycosis were treated with penicillin and have been followed for periods of from one to five years. Of twenty-six patients suffering from cervicofacial actinomycosis, twenty-four had excellent results. These results were obtained after an average period of treatment of less than two months, a period significantly less than the usual length of time required to obtain comparable results when penicillin was not used. Of nine patients who had pulmonary actinomycosis, five recovered. Of eight patients who had abdominal actinomycosis, six recovered. All three patients who had pelvic actinomycosis recovered. All strains of *Actinomyces bovis* cultured from these patients were sensitive to penicillin *in vitro*. Therefore penicillin appears to be an effective chemotherapeutic agent in the treatment of actinomycosis and a useful adjunct to other forms of therapy.

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# MATERNAL ISOIMMUNIZATION WITHOUT EVIDENCE OF CLINICAL ERYTHROBLASTOSIS FETALIS IN THE NEWBORN

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ONE of the many problems which remain to be solved in the field of Rh-Hi isoimmunization or sensitization is why the occasional infant possessing the antigenic factors against which the mother is isoimmunized is born healthy and shows no subsequent evidence of erythroblastosis. Occasionally, in such cases, the concentration of antibodies of both the early immune and hyperimmune type is surprisingly high. An explanation of this phenomenon may possibly furnish a basis for the development of more effective methods of treating the sensitized mother to lessen or abolish the deleterious effects of the antibodies on the fetus.

Instances of normal Rh-positive infants born to mothers isoimmunized against one or more of the factors which the infant has inherited from the father may be divided into two categories:

1. Cases in which the immunization was discovered at or near term with no clear cut history of a previously affected infant.
2. The extremely rare case in which there was a history of a previously affected infant.

Several reports have appeared in the literature of clinically normal Rh-positive infants born to Rh-negative mothers whose blood contained Rh antibodies. In the majority of these reports there was no clear cut history of a previous child suffering from erythroblastosis. Possibly, in these cases, the production of antibodies had been initiated too late in pregnancy to have resulted in significant disease of the fetus or the concentration of antibodies was at no time sufficient to cause fetal damage.

It is probably correct to assume that once isoimmunization against one or more of the Rh-Hi factors has been established, either as a result of pregnancy or transfusion, it is permanent even though the presence of antibodies cannot be detected by present laboratory methods. Experience has shown that in the usual course of events if a mother has been immunized against one or more of the Rh-Hi factors and has borne a child with erythroblastosis, all subsequent infants who inherit one or more of the factors against which the mother is isoimmunized will suffer from erythroblastosis. Furthermore, each succeeding infant is usually affected to a greater degree than its immediate predecessor. Potter,<sup>1</sup> in her monograph, repeatedly substantiates this viewpoint more or less categorically and infers that there are no exceptions to this general rule. She was unable to find any examples in the literature where a normal Rh-positive infant was born to a mother who had previously given birth to an infant suffering from erythroblastosis.

Burnham,<sup>2</sup> Dockeray and Sachs,<sup>3</sup> Goldbloom and Lubinski,<sup>4</sup> and Karher and Miller<sup>5</sup> all have described cases in which apparently normal Rh-positive

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infants were born to mothers who exhibited it or new term Rh antibodies. In none of the reports was there conclusive evidence of a previous erythroblastotic infant.

Demy<sup>6</sup> published an example of severe erythroblastosis in one of Rh positive identical twins, with only mild manifestations in the other. Both of the twins survived. The mother was Rh negative and the father Rh positive. The mother's blood presumably contained antibodies in high titer. The mother's previous obstetric history was as follows: There were four successive normal pregnancies in 1924, 1927, 1929, and 1932. In 1934 she gave birth to twins, one of whom was stillborn, the other lived four hours and died of hemorrhage. In 1936 she gave birth to a sixteen pound stillborn infant and although no data were available, it is presumed that this was a fatal case of fetal hydrops.

Although one might deny that the pregnancies terminating in 1934 and 1936 were concerned with Rh isoimmunization, it is difficult to explain the great difference in the degree of involvement of the second set of twins born of the last pregnancy, both of whom survived. The author postulates a greater functional defect in that portion of the placenta serving the twin with the more severe disease. It would seem in this case that other factors, besides the duration of action, concentration, and type of antibodies, influenced the deleterious effect of the antibodies on the fetal tissues. Assuming that the constitutional susceptibility of the identical twins was the same and that the maternal factors were constant for both twins, one has to conclude, as did Demy, that the explanation for the variation was a functional difference in the portion of the placenta serving each twin. It appears that the placenta may play a significant role in the protection of the fetus even if isoimmunization has been established.

Cappell<sup>7</sup> cites an example (Case 10) which is very suggestive of normal Rh positive children being born after erythroblastosis has been established in earlier offspring. But, as Cappell points out, however suggestive the case history may be, the cause of death in the earlier infants was not fully ascertained since postmortem examinations were not done. Cappell also states that Dr. Stinbury<sup>8</sup> and Dr. Chownt<sup>9</sup> both encountered cases in which a healthy unaffected Rh positive child was born to an Rh negative mother who had previously delivered a child with severe erythroblastosis. The data on the family studied by Stinbury<sup>8</sup> (Case 3), although very suggestive and probably valid, cannot be accepted as definitely proved since the children presumed to have suffered from erythroblastosis antecedent to the normal infant were born prior to the discovery of the Rh factor and it is not stated whether or not a postmortem was performed.

Drumond<sup>10</sup> has seen nine infants who showed no clinical evidence of erythroblastosis, although they were all Rh positive and were born to women who were isoimmunized to the Rh factor which their babies had.

The two cases which we are reporting are definite exceptions to the usual experience. Fortunately the opportunity presented itself to study these families in some detail. We believe that enough data were obtained to prove that a healthy mother may have an erythroblastotic offspring, followed by a healthy infant of

identical or similar Rh pattern. It is felt that this report is important as it establishes the fact that such cases do occur and thus offers some hope that eventually a method of treatment may be discovered whereby the immunized mother can be assured of having a normal Rh-positive baby. As far as we can determine, no unequivocal example similar to ours has yet been published.

CASE 1—Mrs M N, Group O, Rh negative, cde/cde, Mr G N, Group O, Rh positive, CDe/CDe (negative to anti c)

On July 27, 1942, Mrs N was delivered of a full term male infant (P N), Group O, Rh positive, CDe/cde. The infant was said to be normal and healthy but was mildly jaundiced on the second day of life. The jaundice disappeared on the same day without treatment and the child has been perfectly well since. No Rh studies were done on the mother at that time.

On May 30, 1945, Mrs N delivered a Group O, Rh positive (tested with anti D only), full term female infant weighing 6 pounds 12 ounces. Jaundice appeared on the fourth day and became increasingly severe. The infant had peculiar stiffening spells. By the time of admission to the Hospital for Sick Children, on the sixth day of life, the jaundice was extreme. The liver was two fingerbreadths below the right costal margin, but clinically the spleen was not palpated. No nucleated or immature red cells were seen in the blood film. The baby was treated with 100 ml of normal saline and 60 ml of blood of unknown Rh type intravenously. Her condition deteriorated rapidly and respirations ceased seventeen hours after admission, seven days after birth.

The mother's serum, tested six days post partum, showed agglutinating or early immune antibodies against Rh positive cells. Neither titrations nor tests for blocking or hyperimmune type of antibodies were done at this time.

A post mortem examination was performed. The gross findings were severe jaundice, hepatomegaly, splenomegaly, and marked degree of kernicterus of the basal ganglia, moderate edema of the retroperitoneal tissues, and hemorrhages into the lung parenchyma. Microscopically there was a large amount of extramedullary hematopoiesis in the liver, with a lesser amount in the spleen. Numerous macrophages containing blood pigment were present in the liver and spleen. The findings were in all respects typical of erythroblastosis fetalis (icterus gravis).

On May 7, 1946, Mrs N delivered a male full term infant (F N), Group O, Rh positive, CDe/cde. The infant was seen within a few hours of birth by a pediatrician who has a particularly wide experience with erythroblastosis and who, in view of the mother's past history, was expecting the baby to be severely affected. Throughout the infant's normal stay in the maternity hospital there was at no time any clinical evidence of erythroblastosis. The subsequent progress of the child has been normal.

The mother's serum, tested May 16, 1946, nine days post partum, showed blocking or hyperimmune antibodies only, to a dilution of 1:32 against both CDe and cDe cells. Breast milk tested at that time showed the presence of weak antibodies in 1:2 dilution.

Mrs N's serum was again tested January 18, 1947, eight and one half months post partum, and again only blocking or hyperimmune type of antibodies was present to a dilution of 1:4 against both CDe and cDe cells.

CASE 2—Mrs L, Group O, Rh positive, CDe/CDe, Mr L, Group A, Rh positive, cDe/cde \*

On March 21, 1940, Mrs L delivered a male infant (H L), Group O, Rh positive, CDe/cDe. The infant survived and was normal. Following delivery the mother required two transfusions. One donor, a brother-in-law, was subsequently found to be Group O, Rh positive, CDe/cDe, and the other was Group O, Rh positive, cDe.

On February 25, 1944, Mrs L gave birth to another boy (I L), Group A, Rh positive, CDe/cDe. The infant survived and was normal.

\*Predicted by Dr Louis K Diamond with his anti d serum and subsequently proved by the last child.

On April 1, 1946, the third boy (R L) was born, Group O Rh positive, CDe/cDE. Jaundice was noted at birth and gradually became more severe. Two days after birth the infant was transferred to The Hospital for Sick Children. Examination on admission revealed a severely jaundiced baby with a palpable liver and spleen. The red blood count was 4,300,000 and the hemoglobin 11.9 grams. Blood films showed many polychromatic red cells and 18 normoblasts per 100 white blood cells. The baby received a total of 465 ml of Group O Rh negative blood\* in 14 transfusions over a seventeen day period. Plasma and calcium gluconate were also used in the treatment. On discharge at 19 days of age with a hemoglobin of 13.9 grams, the baby appeared cured. Although the mother states the child was physically slightly retarded at first in comparison with her other children, subsequent development has been normal.

The third baby was considered to have been a clinical case of erythroblastosis. The antibodies demonstrated in the mother were anti E both early immune and hyperimmune and anti c hyperimmune only. Antibody determinations on the mother's serum following the birth of this baby and prior to her last pregnancy were as shown in Table I.

TABLE I. ANTIBODY TITER, MRS L'S SERUM USING KNOWN CELLS

DATE	CDe/CDe		cDe/cDe		INFANT (R L) CDe/cDE		cDe/cDe	
	SALINE	ALBUMIN	SALINE	ALBUMIN	SALINE	ALBUMIN	SALINE	ALBUMIN
4/7/46	0	0	1:32	1:256	0	1:256	-	-
5/3/46	0	0	1:64	1:128	-	-	0	1:4

In January, 1947, it was learned that Mrs L was again pregnant and two samples of blood were obtained prior to her delivery on March 6, 1947. The fourth baby was a full term male Group O, Rh positive CDe/cDe. Because the child was expected to have a case of erythroblastosis, he was admitted to The Hospital for Sick Children one half hour after delivery and followed closely throughout his eight day stay in the hospital. Results of physical examination, red blood count, hemoglobin, and blood film were normal at all times. On admission the hemoglobin was 22 grams. This fell gradually until the day of discharge when it was 16.7 grams. No treatment was given and the subsequent course and development of the child were normal.

TABLE II. ANTIBODY TITER, MRS L'S SERUM USING KNOWN CELLS

DATE	CDe/cDe		cDe/cDe		CDe/CDe		cDe/cDe	
	SALINE	ALBUMIN	SALINE	ALBUMIN	SALINE	ALBUMIN	SALINE	ALBUMIN
1/17/47	0	0	-	-	0	1:8	0	1:4
2/11/47	-	-	0	1:4	0	1:64	0	1:8
7/18/47	0	0	-	-	±	1:16	0	1:4

Undiluted cord serum and the infant's serum on admission exhibited weak antibodies against Rh positive CDe/cDE and Rh negative cells. The infant's cells also gave a 4 plus Coombs test.

Antibody determinations during and following Mrs L's last pregnancy are shown in Table II. Unfortunately, blood from the mother in the immediate post partum period was not available.

## COMMENT

It is considered that Case 1 is a clear cut example of a normal Rh positive child being born to an isosensitized Rh negative mother subsequent to the

Preferably this infant should have received CDe/CDe blood but at the time of the child's admission facilities were not available for full investigation of the case or Rh subtyping of the donor's blood.

birth of an infant which suffered from erythroblastosis severe enough to cause death. Agglutinating Rh antibodies were demonstrated following the birth of the second child, and postmortem revealed the cause of death of this child to be erythroblastosis. Rh antibodies of the hyperimmune variety were present following the birth of the last child which was Rh positive and clinically normal. Any explanation in the light of our present knowledge must be pure conjecture. If one believes that fetal erythrocytes must cross the placental barrier to induce isoimmunization, a possible explanation, in view of the relatively low titer of antibodies (1:32) present after the birth of the third and normal child, is that during the third pregnancy the placental barrier was intact at all times and that no fetal cells gained entrance to the maternal circulation to further stimulate the production of antibodies to a sufficient level to cause fetal damage. Against this theory is the fact that the titer is not a reliable index of the amount of damage antibodies may cause. Frequently we have encountered persistently low antibody titers, as determined by the ordinary laboratory methods for the agglutinating and blocking of hyperimmune types, in cases where the infant was stillborn or severely involved.

The circumstances in Case 2 are such that a tentative explanation for the normality of the last baby is possible. The isoimmunization of this Rh positive, CDe/CDe mother which is of a relatively rare type, is a manifestation of an antibody response to two antigens, E and e. If the relative frequency of the occurrence of antibodies is any criterion, E and e must be considered as weak antigens. The necessary Rh and H<sub>1</sub> factors to stimulate the production of the antibodies found in the mother's serum were present in the children of the first three pregnancies all of which were CDe/cDE, and in the two donors, who were CDe/cDE and cDE respectively. Presumably three pregnancies and two transfusions were necessary to stimulate antibody production to a sufficient level to result in a moderately severe case of erythroblastosis. It is possible that the isoimmunization was initiated by the two transfusions following the first pregnancy but the antibody concentration did not reach a sufficient level during the second pregnancy to result in clinical erythroblastosis in that child. During the third pregnancy both anti-E and anti-e were effective against the fetal erythrocytes, the former apparently being the more potent as judged by the titrations. Probably the combined effect of these antibodies, particularly anti-E, was sufficient to result in clinical disease in the infant. In the last pregnancy, resulting in an Rh-positive, CDe/cde boy, only anti-e was effective against the fetal erythrocytes. Although antibodies could be demonstrated in the infant's serum and adsorbed to his erythrocytes, apparently this one effective antibody was not present in sufficient concentrations to have resulted in any appreciable damage to the infant's red blood cells.

#### SUMMARY

Two cases are reported of maternal Rh-H<sub>1</sub> isoimmunization which resulted in infants suffering from erythroblastosis, in each case the mother, in a subsequent pregnancy, delivered a clinically normal baby possessing at least one Rh-H<sub>1</sub> factor against which she was isoimmunized.



We wish to acknowledge the kindness of Dr Louis K Diamond, Boston, Mass, for providing us with specific Rh antisera and for checking our results. We are also much indebted to Mrs E M Hutchinson for her able technical assistance.

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## ANEMIA OF INFECTION

### IX INFLUENCE OF ADRENALECTOMY AND OF ADRENAL CORTICAL HORMONE ON HYPOFERREMIA AND OTHER BLOOD CHANGES ASSOCIATED WITH INJECTION OF TURPENTINE

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OF THE profound metabolic changes accompanying infection, one which seems to be related to the anemia associated with infection is hypoferrinemia.<sup>1</sup> In view of the role which the adrenal gland has been shown to play in conditions of stress,<sup>2</sup> it seemed desirable to determine whether or not this gland is concerned with this state of hypoferrinemia. Since a marked fall in plasma iron occurs following injection of either turpentine or staphylococci,<sup>3</sup> the former was adopted as the more easily controllable means for producing the hypoferrinemia of inflammation. In addition to observations on the plasma iron in normal and adrenalectomized rats following intramuscular injections of turpentine, changes in hemoglobin and in the total and differential white cell counts are recorded.

#### MATERIALS AND METHODS

One hundred twenty eight albino rats of both sexes, of Sprague Dawley strain, each weighing between 100 and 200 grams, were used in this study. The animals were divided into several groups as described under Results. Bilateral adrenalectomy was performed by the dorsal route as described by Griffith and Farris,<sup>4</sup> care being taken to avoid rupture of the capsule and to control bleeding during operation. Following adrenalectomy, these animals were allowed 1 per cent sodium chloride as fluid. The diet otherwise was the same as that of the controls. After seven days, data were secured to establish the range of values for adrenalectomized rats.

Originally it was intended to inject 0.5 ml of turpentine in normal and in adrenalectomized rats. Unlike the normal controls, the adrenalectomized rats could not withstand this dose of turpentine. But three out of seventeen survived for four hours. By a process of trial and error, a dose of 0.1 ml of turpentine per 100 grams body weight was selected as one which could be given to adrenalectomized rats as well as to normal unadrenalectomized rats.

Blood for estimation of hemoglobin and total and differential leucocyte counts was obtained from the tail veins of the animals. For plasma iron determinations, groups of three or four were anesthetized with 0.5 ml of 1 per cent Nembutal per 100 grams body weight in the case of normal intact rats and with 0.4 ml per 100 grams in the adrenalectomized rats. When fully anesthetized, their abdomens were opened and blood was collected from the abdominal aorta in iron free syringes and pooled in iron free centrifuge tubes. Estimation of plasma iron was made from the pooled blood by the method of Barkan and Walker.<sup>5</sup> Hemoglobin was determined photoelectrically with Evelyn's colorimeter.<sup>6</sup> Total and differential white cell counts were done by the usual techniques, cover slip preparations being used for the latter purpose.

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## RESULTS

All data, save for some of the values for plasma iron which consisted of single observations, have been examined by Fisher's *t* test. Mamlund<sup>8</sup> has pointed out that this is the only valid method for estimating the significance of means when the number of observations in the series is thirty or less.

*Observations in Twenty One Normal and Fifteen Adrenalectomized Rats* — Data for normal levels of hemoglobin, for total and differential white cell counts and for plasma iron in normal and adrenalectomized rats are presented in Table I. The values for iron were somewhat lower than had been observed in normal rats in previous experiments in this laboratory.<sup>9</sup> Although monocytes and eosinophiles were encountered in the differential counts values for these have not been entered in the table as they generally were few in number as compared with other cells and nothing striking was found about them. Table I also gives the comparison of the mean values by the *t* test already referred to. Comparing the values of *t* with those at the usually accepted level of significance (that is, probability of 5 per cent) it is seen that a significant increase

TABLE I. COMPARISON OF BLOOD VALUES IN NORMAL AND ADRENALECTOMIZED RATS

		VALUES IN NORMAL RATS	VALUES IN ADRENALEC TOMIZED RATS	DIFFER ENCE IN THE RE SPECTIVE MEAN VALUES OF THE TWO GROUPS	STANDARD ERROR OF THE DIFF ERENCE OF MEAN VALUES	VAL UES OF <i>t</i>	RE MARKS
Plasma iron ( $\mu$ g per 100 ml)	Number of ob servations	4	5				
	Mean	202	166	36	$\pm 36$	1.00	Not sig nifi cant
	Range	121-262	133-252				
	Standard deviation	$\pm 59$	$\pm 49$				
Hemoglobin (Gm per 100 ml)	Number of ob servations	14	13				
	Mean	13.91	14.94	+1.03	$\pm 0.39$	2.64	Signifi cant
	Range	12.69-15.15	12.98-16.80				
	Standard deviation	$\pm 0.77$	$\pm 1.24$				
Total white cells (per c.mm)	Number of ob servations	16	14				
	Mean	18,175	20,289	+2,114	$\pm 2,070$	1.02	Not sig nifi cant
	Range	10,950-31,000	12,600-29,600				
	Standard deviation	$\pm 6,100$	$\pm 5,103$				
Neutro philes (per c.mm)	Number of ob servations	16	14				
	Mean	3,502	7,253	+3,691	$\pm 969.2$	3.81	Signifi cant
	Range	1,403-6,442	2,520-13,170				
	Standard deviation	$\pm 1,743$	$\pm 3,463$				
Lympho cytes (per c.mm)	Number of ob servations	16	14				
	Mean	14,021	12,266	-1,755	$\pm 1,385$	1.37	Not sig nifi cant
	Range	8,586-23,481	7,923-14,800				
	Standard deviation	$\pm 4,450$	$\pm 2,123$				

Calculated by dividing the difference in the mean values by their standard errors

in hemoglobin and in neutrophiles was observed following adrenalectomy but no significant changes could be demonstrated in total white cell count, lymphocytes, or plasma iron

*Observations on the Effects of Injection of Turpentine in Normal and Adrenalectomized Rats —*

1 In a series of twenty one rats, irrespective of individual weights, 0.5 ml of turpentine was injected in the leg. Blood studies were made at intervals of four, eight, twelve, twenty-four, forty-eight, and ninety six hours after injection. Data for plasma iron and hemoglobin obtained from these animals, collected in Table II, show a rise in hemoglobin level reaching its maximum at twelve

TABLE II FLUCTUATIONS IN LEVEL OF PLASMA IRON AND HEMOGLOBIN IN RATS INJECTED WITH 0.5 ML OF TURPENTINE, AND SIGNIFICANCE OF HEMOGLOBIN VALUES AS COMPARED WITH THOSE FOR NORMAL UNTRAUMATIZED RATS

HOURS AFTER INJECTION	PLASMA IRON ( $\mu\text{G}/100 \text{ ML}$ )	HEMOGLOBIN		
		GM PER 100 ML	VALUES OF "T"	REMARKS
4	175.54	16.29	3.45	s
8	117.90	16.94	6.06	
12	73.70	17.69	7.56	
24	47.02	15.74	3.81	
48	152.96	13.95	0.09	n s
96	159.89	15.20*	2.32	

\*Mean of two observations only

s Significant n s not significant

hours and a fall in plasma iron reaching its lowest level at twenty four hours. A sudden drop of total leucocytes and lymphocytes, accompanied by a slower drop in granulocytes, is evident from Fig 1, where the values for these have been charted. The significance of these values as compared with data for normal untraumatized controls is indicated in Table III.

TABLE III FLUCTUATION IN VALUES FOR WHITE CELLS IN RATS RECEIVING 0.5 ML. OF TURPENTINE AND THEIR SIGNIFICANCE AS COMPARED WITH VALUES FOR NORMAL UNTRAUMATIZED RATS

HOURS AFTER INJECTION	TOTAL WHITE CELLS			LYMPHOCYTES			NEUTROPHILES		
	NUMBER PER C MM	VALUE OF "T"	REMARKS	NUMBER PER C MM	VALUE OF "T"	REMARKS	NUMBER PER C MM	VALUE OF "T"	REMARKS
4	11,583	1.82	n s	5,987	3.05	s	5,497	1.80	n s
8	6,667	3.18	s	4,546	3.98		1,889	1.71	
12	3,100	4.18		1,985	4.58		961	2.67	
24	7,567	2.92		5,721	3.12		1,614	2.01	
48	12,500	1.55	n s	9,783	1.58	n s	2,240	1.35	n s
96	15,700*	0.66	n s	10,889	0.96	n s	4,217	0.54	

\*Mean of two values only

s Significant n s not significant

2 In a series of twenty-four normal intact rats turpentine was injected in a dose of 0.1 ml per 100 grams. Data from these rats are summarized in Tables IV and V. As can be seen from Fig 2, with this dose normal intact rats reacted in essentially the same manner as those receiving 0.5 ml of turpentine. The leucocyte curves exhibited more or less the same features as those of rats receiving 0.5 ml of turpentine, the drop in values was, however, less

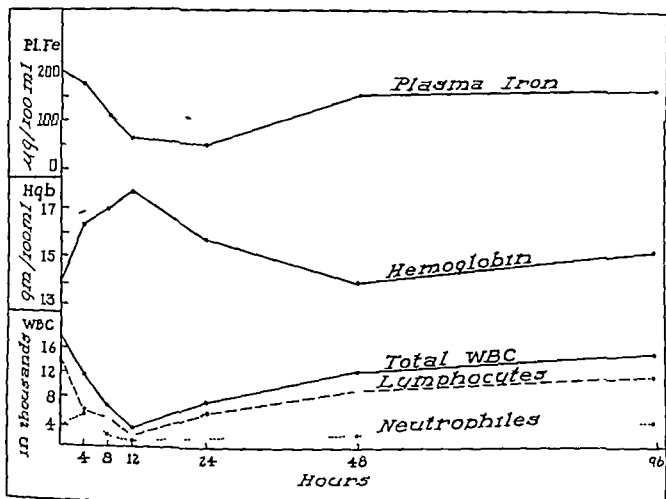


Fig 1—Changes in plasma iron hemoglobin and white cells in normal intact rats receiving 0.5 ml of turpentine injected at 0 hour

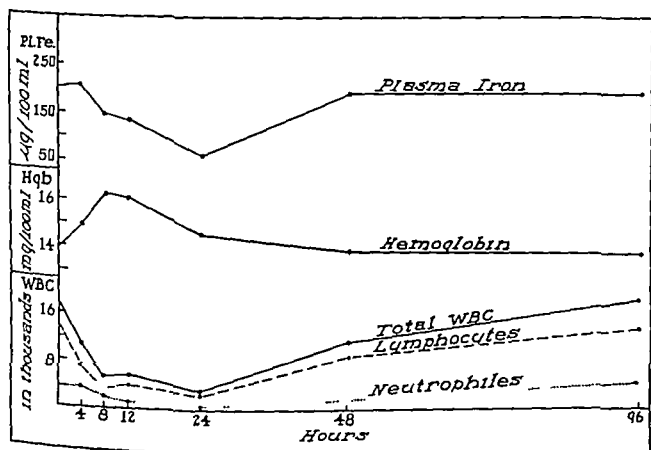


Fig 2—Changes in plasma iron hemoglobin, and white cells in normal intact rats receiving 0.1 ml of turpentine per 100 grams injected at 0 hour

TABLE IV FLUCTUATIONS IN LEVEL OF PLASMA IRON AND HEMOGLOBIN IN RATS INJECTED WITH 0.1 ML OF TURPENTINE, AND SIGNIFICANCE OF HEMOGLOBIN VALUES AS COMPARED WITH THOSE FOR NORMAL UNTRAUMATIZED RATS

HOURS AFTER INJECTION	PLASMA IRON ( $\mu\text{G}/100 \text{ ML}$ )	HEMOGLOBIN		
		GM PER 100 ML	VALUES OF "T"	REMARKS
4	208.73	14.92	1.70	ns
8	148.51	16.27	5.13	
12	137.23	16.15	4.76	s
24	66.00	14.67	1.61	
48	195.30	13.97	0.13	ns
96	188.15	13.83	0.17	

s Significant ns not significant

precipitous, the lowest value being delayed until twenty-four hours after injection. The significant drop in leucocytes occurred at practically the same period, as will be seen from Table V.

TABLE V FLUCTUATION IN VALUES FOR WHITE CELLS IN RATS RECEIVING 0.1 ML OF TURPENTINE AND THEIR SIGNIFICANCE AS COMPARED WITH VALUES FOR NORMAL UNTRAUMATIZED RATS

HOURS AFTER INJECTION	TOTAL WHITE CELLS			LYMPHOCYTES			NEUTROPHILES		
	NUMBER PER CMM	VALUE OF "T"	REMARKS	NUMBER PER CMM	VALUE OF "T"	REMARKS	NUMBER PER CMM	VALUE OF "T"	REMARKS
4	10,967	1.99	ns	7,095	2.61	s	3,688	0.13	ns
8	5,517	3.50	s	3,277	4.08		2,182	1.41	
12	5,750	3.43		4,119	3.74		1,532	2.08	
24	3,583	4.04		2,713	4.29		794	2.85	s
48	11,833	1.72	ns	9,425	0.88	ns	2,019	1.58	ns
96	18,700	0.13		13,735	0.10		4,577	0.97	

s Significant ns not significant.

3. In twenty-four adrenalectomized rats 0.1 ml of turpentine per 100 grams weight was injected and data collected as before. As is evident from Fig. 3, although there was the same tendency toward a fall in plasma iron and a rise in hemoglobin curve within the first twelve hours as in traumatized unadrenalectomized rats, the subsequent changes differed markedly. Thus Table VI brings out the fact that from twenty-four hours and on after injection, the hemoglobin in these animals seemed to be stabilized at a significantly lower level than before injection and, concurrently, there was a definite lag in the rise of the plasma iron curve.

TABLE VI FLUCTUATIONS IN LEVEL OF PLASMA IRON AND HEMOGLOBIN IN ADRENALECTOMIZED RATS RECEIVING 0.1 ML OF TURPENTINE, AND SIGNIFICANCE OF HEMOGLOBIN VALUES AS COMPARED WITH THOSE FOR UNTRAUMATIZED ADRENALECTOMIZED RATS

HOURS AFTER INJECTION	PLASMA IRON ( $\mu\text{G}/100 \text{ ML}$ )	HEMOGLOBIN		REMARKS
		GM PER 100 ML	VALUES OF "T"	
4	150.65	14.86	0.10	ns
8	117.22	16.17	1.32	
12	52.76	15.52	0.73	
24	69.08	12.63	2.88	s
48	88.58	13.22	2.24	
96	96.02	12.61	2.87	

s Significant ns not significant

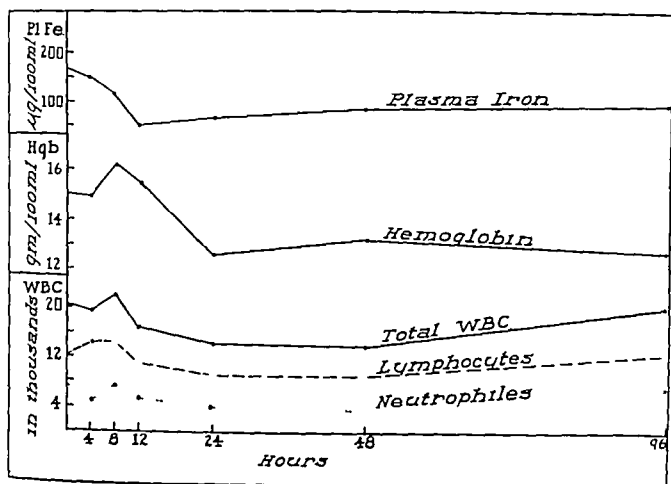


Fig 3—Changes in plasma iron hemoglobin and white cells in adrenalectomized rats receiving 0.1 ml of turpentine per 100 grams injected at 0 hour

With regard to the leucocyte picture, there was a marked departure from the pattern observed in all the previous groups in that no significant alterations took place (Fig 3, Table VII)

TABLE VII FLUCTUATIONS IN VALUES FOR WHITE CELLS IN ADRENALECTOMIZED RATS RECEIVING 0.1 ML OF TURPENTINE AND THEIR SIGNIFICANCE AS COMPARED WITH THOSE FOR UNTRAUMATIZED ADRENALECTOMIZED RATS

HOURS AFTER INJE- CTION	TOTAL WHITE CELLS			LYMPHOCYTES			NEUTROPHILES		
	NUMBER PER C MM	VALUE OF 'T'	REMARKS	NUMBER PER C MM	VALUE OF 'T'	REMARKS	NUMBER PER C MM	VALUE OF 'T'	REMARKS
4	19 267	0.20	ns	14 234	1.19	ns	4 874	1.17	ns
8	22,167	0.61		14 384	1.64		7 532	0.12	
12	16,817	1.14		11 078	0.76		5 365	0.82	
24	14,284	1.90		9,750	1.74		4 134	1.43	
48	13 933	2.08		9 750	1.99		3 828	1.65	
96	20 300	0.00		12 720	0.30		6 940	0.14	

ns. Not significant

*Observations on the Effects of Cortical Hormone on Normal Rats*—In a series of twenty intact normal rats adrenal cortical extract\* was injected intramuscularly in doses of 10 ml per rat without regard to individual weights. This dose is double that used by Dougherty and White<sup>10, 12</sup> in their experiments and was chosen advisedly in order to emphasize the effects if any of cortical hormone on the level of plasma iron in rats. As is evident from Fig 4, there

\*Adrenal cortical extract was furnished by the Upjohn Company, Kalamazoo, Mich.

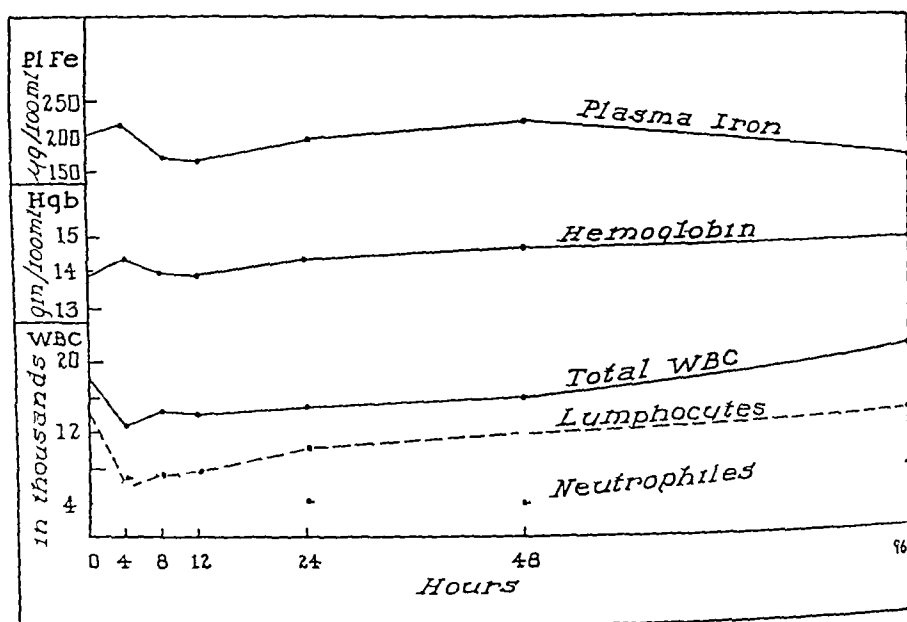


Fig 4—Changes in plasma iron hemoglobin and white cells in normal intact rats receiving 10 ml of cortical hormone injected at 0 hour

TABLE VIII FLUCTUATIONS IN LEVEL OF PLASMA IRON AND HEMOGLOBIN IN RATS RECEIVING 10 ML OF CORTICAL HORMONE, AND SIGNIFICANCE OF HEMOGLOBIN VALUES AS COMPARED WITH THOSE FOR NORMAL RATS NOT RECEIVING SUCH INJECTIONS

HOURS AFTER INJECTION	PLASMA IRON ( $\mu\text{G}/100 \text{ ML}$ )	HEMOGLOBIN		
		GM PER 100 ML	VALUES OF "T"	REMARKS
4	217.34	14.42	1.10	ns
8	172.71	14.01	0.21	
12	166.81	13.96	0.10	
24	196.81	14.39*	0.86	
48	222.39	14.68	1.49	
96	172.71	14.86	2.02	

\*Mean of two observations only

ns Not significant

TABLE IX FLUCTUATIONS IN NUMBER OF WHITE CELLS IN RATS RECEIVING 10 ML OF CORTICAL HORMONE AND THEIR SIGNIFICANCE AS COMPARED WITH VALUES FOR NORMAL RATS NOT RECEIVING SUCH INJECTIONS

HOURS AFTER INJECTION	TOTAL WHITE CELLS			LYMPHOCYTES			NEUTROPHILES		
	NUMBER PER CMM	VALUE OF "T"	REMARKS	NUMBER PER CMM	VALUE OF "T"	REMARKS	NUMBER PER CMM	VALUE OF "T"	REMARKS
4	12,733	1.50	ns	5,657	3.11	s	6,777	3.14	s
8	14,300	1.08		6,992	2.67		7,123	3.08	
12	14,167	1.10		7,106	2.62		6,902	2.97	
24	14,633	0.96		10,110	1.47	ns	4,206	0.69	n
48	15,883	0.63		11,746	0.86		3,793	0.23	
96	21,033	0.77		13,650	0.13		7,274	3.59	

s Significant ns not significant.



did not seem to be much variation in the levels of plasma iron or hemoglobin. This impression is confirmed also by examination of Table VIII where the values for these rats have been summarized.

The behavior of the leucocytes was very striking however, as is evident from Fig. 4. Within four hours after injection there was a fall in the number of lymphocytes, with a corresponding increase in the number of neutrophils as well as some variation in the total white cells. The significance of these variations is given in Table IX.

#### DISCUSSION

Neither adrenalectomy nor injection of cortical hormone produced any significant change in the value of plasma iron in normal rats. On the other hand, both in normal intact and in adrenalectomized rats the injection of turpentine was immediately followed by a significant drop in the level of plasma iron. Thus hypoferrinemia following turpentine injection is independent of the presence or absence of the adrenal glands in rats. However the hypoferrinemia seemed to persist longer in adrenalectomized than in nonadrenalectomized rats. Further, in adrenalectomized rats, this prolonged hypoferrinemia was associated with a significantly lower level of hemoglobin. It appears, therefore, that the adrenal gland may be related in some way to the quick recovery from hypoferrinemia which occurs in intact rats injected with turpentine and to the maintenance of the normal hemoglobin level.

Adrenalectomy was followed by a significant rise in hemoglobin values in rats. This was probably due to hemoconcentration which is known to occur in most animals after adrenalectomy. No significant change in hemoglobin was noticed in normal rats on injection of cortical hormone. The immediate effect of turpentine injection on the hemoglobin values of both normal and adrenalectomized rats was a sudden rise. This was more marked in the normal than in the adrenalectomized rats.

The number of total leucocytes was not significantly altered either by adrenalectomy or by injection of cortical hormone in normal rats. Our findings, here, are not in agreement with those of Dougherty and White<sup>10, 12</sup> in whose hands there was an increase following adrenalectomy and a decrease on injection of cortical extract. Corey and Britton<sup>13</sup> observed a fall in leucocytes in rats following adrenalectomy. The effect of turpentine injection on the total leucocytes of normal intact rats and adrenalectomized rats differed markedly. In the normal nonadrenalectomized rats there was a sharp and significant drop in leucocytes, in the adrenalectomized rats, on the contrary, no such significant fall was noticed. It seems, therefore, that the adrenal gland may be in some way connected with the peripheral leucopenia that occurs in normal rats following turpentine injection.

Both adrenalectomy and the injection of cortical extract in normal rats caused a significant rise in the number of neutrophils. These results are difficult to interpret. The turpentine injection caused a significant drop of neutrophils in normal rats but no such effect was observable in adrenalectomized rats.

In our hands, adrenalectomy did not significantly alter the number of lymphocytes. The injection of cortical hormone in normal rats, however, produced a significant drop in the number of lymphocytes and this finding is in accord with that of Dougherty and White<sup>10-12</sup>. The effect of turpentine injection on the lymphocytes was the same as on neutrophils inasmuch as it produced a significant drop in the normal intact animals but none at all in adrenalectomized rats.

From the foregoing, it is clear that although the adrenal gland does not affect hypoferrremia as such, its presence may possibly be necessary for the quick recovery from hypoferrremia as well as for the delay in the onset of anemia such as occurs from turpentine injection. The adrenal gland may also be involved in the leucopenia which follows the injection of turpentine.

#### SUMMARY AND CONCLUSION

A series of one hundred twenty-eight albino rats was used in this study. Of these, twenty one served as normal controls, forty-five received injections of turpentine in two separate groups in doses of 0.5 and 0.1 ml, respectively, while twenty were injected with adrenal cortical extract. Of the remaining forty-two, which were all adrenalectomized, fifteen served as controls, twenty four received 0.1 ml and three 0.5 ml of turpentine. Plasma iron, hemoglobin, total and differential white cell counts were determined in these animals. Information regarding the original data has been condensed into a number of tables and the significance tested.

The following conclusions have been reached:

- 1 The adrenal gland has no effect on the normal level of plasma iron in rats, nor does it affect the hypoferrremia which results in these animals from turpentine injection.
- 2 The adrenal gland may, however, be related in some way to the quick recovery from hypoferrremia induced by the injection of turpentine.
- 3 The adrenal gland does not seem to have any influence on the maintenance of the normal hemoglobin level in rats, though under conditions of stress, such as turpentine injection, it may play some role in preventing a drop in the level of hemoglobin that might otherwise occur.
- 4 The adrenal gland is intimately concerned with the peripheral leucopenia that follows turpentine injection in normal rats. This leucopenia involved both lymphocytes and granulocytes and was not observed in adrenalectomized rats.

The guidance of Dr. George Sayers in performing the adrenalectomies is gratefully acknowledged.

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## IDIOPATHIC (FAMILIAL) HYPOPROTHROMBINEMIA

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RHOADS and Fitz-Hugh in 1941<sup>1</sup> first recorded an instance of idiopathic hypoprothrombinemia as the basis for an hemorrhagic diathesis. Since then eight\* additional cases have been reported<sup>2, 6, 11</sup>. Hypoprothrombinemia has been noted in several individuals otherwise normal or in persons who have had only one significant bleeding episode<sup>3a, 4, 6, 10</sup>. Familial hypoprothrombinemia has been demonstrated in at least four instances<sup>2, 5, 6, 10</sup>.

Most of the patients thus far reported were not followed in detail over a long period of years. Some reports lack sufficient data to establish beyond doubt that the prolonged prothrombin time is due to prothrombin deficiency. Table I summarizes the cases of idiopathic hypoprothrombinemia from the literature.

The patient who formed the basis for the present study was seen repeatedly since December, 1937, on various services at the University Hospital. Besides numerous outpatient visits there were forty-seven hospital admissions. During this period a considerable mass of clinical and laboratory data was collected. The purpose of the present report is to summarize these observations, to describe recent and more detailed studies of the patient's abnormality, and to compare the information gained with that contained in the literature.

### CASE REPORT

J. L., a 20 year old female domestic, first visited the University Hospital Out Patient Department in December, 1937, for study and treatment of an eight year bleeding tendency.

*Past Health*—Birth and neonatal periods, as well as subsequent development, were entirely normal. The patient had the usual childhood diseases, but except for the hemorrhagic tendency her past health was good.

*Family History*—The parents were of Swedish extraction. Their families had no blood relatives in common. The mother's paternal grandmother was said to be a bleeder. The mother had always bruised easily but had never had abnormal bleeding. No one else in the patient's ancestry was known to have a hemorrhagic tendency. The patient's siblings were normal except that the youngest, now 13 years old, had severe melena at 2 weeks of age. One brother (R) had severe rheumatic fever. Detailed studies of the immediate family will be given.

*Present Illness*—Nothing abnormal was observed until the child was 2 years of age when she developed a swollen, discolored, painful left knee apparently without trauma. From that time on the course was characterized by recurrent episodes of various painful joint epistaxis (up to the menarche), ecchymotic areas and deep, painful hematomas, both apparently spontaneous and obviously posttraumatic, persistent bleeding from lacerations, a few showers of petechiae especially over the buttocks, abdominal pain, and menorrhagia and metrorrhagia from the onset of menses until a recent hysterectomy. It would be difficult to say that the patient had true hemarthrosis. The joints usually were not swollen, blood was not aspirated, and no limitation of motion of any joint resulted. There never was significant hematuria.

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\*Beard's case cited by Quick\*\* is not included since recent study\*\* showed no hypoprothrombinemia

TABLE I CASES ILLUSTRATING IDIOPATHIC HYPOFIBRINOGENEMIA

PATIENT	ALIQUOT BY	AGE	HISTORY	FAMILY HISTORY	HEMATOLOGIC STUDIES	FIBRINOGEN TIME AND QUALITATIVE RESPONSE
1	Rhoads and Fitz Hugh	1941	♂ 18 yr Onset at 3 months with probable hemarthrosis right knee course—repeated tooth socket and gum hemorrhages, prolonged bleeding from cuts and lacerations hematomas, hemarthrosis and hematuria 26 hospital admissions due to cerebral hemorrhage	None for any abnormal hemorrhagic tendency	BT (Duke) ~ 20 min, often normal, CT (Larsen) 5-360 min, almost always prolonged CR variable 1 G normal TT negative once positive once (-5 p. technique) normal quantity, but qualitative defect believed present anticoagulant behaved ruled out	Quick 70-100 sec controls 70-100 sec refractory to vitamin K blood apparently had hemostatic effect
2	Giordano	1943	♂ 22 yr Onset at 5 years with frequent, spontaneous epistaxis, course—recurrent epistaxis, tooth socket and gum hemorrhages hemarthrosis, at times patient noted skin petechiae and moderate icterus	Both parents and 2 siblings hypofibrinogenemic, maternal aunt and sister known bleeders, parents and 2 siblings had positive TT, father's BT 11½ min, all had elevated serum proteins (0.34-5.99 gm %)	BT 7½ and 4 min, CT CR and PC normal TT positive, T 0.48 Gm % anticoagulant study not specifically mentioned (probably not present addition of pituitary plasma to control plasma did not prolong TT beyond that expected from dilution)	Smith, 9% of normal, refractory to vitamin K blood had apparent hemostatic effect, plasma increased prothrombin in vivo
3	Lums	1943	♀ 40 yr Apparently normal individual hypofibrinogenemia detected incidentally however, a few times had strong and long lasting nosebleeds	No information given (Hemidise a report brother had severe epistaxis nephews have fibromyositis and hypofibrinogenemia)	CF spontaneous coagulation of recalcified blood (Larsen and Lums) 0-2 sec (normal 1.0-1.0 sec) CR and 1 G normal TT negative, 1 204 Gm % (gram)	Larsen and Lums 30 sec, control, 18 sec refractory to vitamin K
4	Phumisa	1943	♂ 18 yr Normal individual and hypofibrinogenemia detected incidentally	No information given	CT spontaneous coagulation of recalcified blood (Larsen and Lums) 207 sec (normal 120-150 sec) 1 G normal TT negative	Larsen and Lums, 28 sec, control 18 sec refractory to vitamin K

BT Bleeding time CT clot retraction PC platelet count TT tourniquet test for capillary fragility I fibrinogen  
 (Cont'd on next page)

TABLE I—CONT'D

PATIENT	REPORTED BY	YEAR	HISTORY	FAMILY HISTORY	HAEMATOLOGIC STUDIES	PROTHROMBIN TIME AND HEPATIC FUNCTION
5	Quick <sup>4,6</sup>	1943	♂, 22 yr Normal individual, hypoprothrombinemia detected incidentally	Mother and sister hypoprothrombinemic (a little lower than 50%), sister has had several episodes of uterine bleeding	BT normal, CT 5.6 min, CR and PC normal, F <sub>2</sub> quantitatively normal, antieagulants believed ruled out	Quick, 45% of normal, deficiency of Quick's component B refractory to vitamin K
6	Murphy and Clark <sup>7</sup>	1941	♂, 18 yr Onset at 4 years with profuse, persistent epistaxis following traumatic fracture of nose, course—recurrent epistaxis, gum hemorrhages, one episode of hematuria	Sister died at 5 years following severe, recurrent epistaxis, father slightly hypoprothrombinemic (PT, Quick, 19 sec, control, 16 sec) (authors believed this was probably of no significance)	BT (Duke) 1½, 15 min, more frequently normal, CT, CR, and PC normal, PT negative, macroscopic examination of nasal bed capillaries revealed "definitely abnormal and bizarre forms", F 0.331 Gm %, "definitely suggestive evidence of some type of qualitative defect", antieagulants believed ruled out	Quick, 60-100 sec, control, 16 sec, refractory to vitamin K, blood found to have hemostatic effect
7	de Murval and Bouché <sup>8</sup>	1944	♀, 14 yr Onset of epistaxis at 8 years, course—recurrent epistaxis, then gum hemorrhages at 14 years	Parents distantly cousins, guineous, no bleeders known	BT normal, CT 12.40 min, CR and PC normal, TT slightly positive at times, negative at times, F 0.56 Gm %	Quick, 25-53% of normal, refractory to vitamin K
8	de Murval <sup>9</sup>	1945	♀, 23 yr Onset at 3 years with profuse bleeding from dog bite, course—recurrent tooth socket and gum hemorrhages, hemarthrosis, menorrhagia, one normal pregnancy, one paratuberculous (abnormal hemorrhages at parturition)	Parents were cousins, father died at 39 of an hemorrhagic disease, mother normal, but had many spontaneous abortions, four of 9 brothers died in infancy, cause unknown	BT normal, CT 10.12 min (author indicates that these levels are prolonged), CR and PC normal, TT negative	Quick, 20-25% of normal, refractory to vitamin K



The patient was first admitted to the University Hospital in March, 1938, for various studies. From then until the onset of menstruation in January, 1941, there were three admissions for epistaxis or prolonged bleeding from a laceration. From the menarche until hysterectomy, in September, 1945, there were thirty seven admissions concerned primarily with the uterine bleeding. Moreover, the other symptoms mentioned recurred over this period.

After the hysterectomy there were four hospitalizations here and one elsewhere. Two were for exacerbations of ecchymoses, painful deep hematomas, and various joint aches, while three admissions were for probable intra abdominal hemorrhage. Pelvic hematomas were present on two occasions. The last admission was in March, 1947.

Various studies were made and a variety of medications was tried during these hospitalizations. However, no cause for the bleeding other than hypoprothrombinemia was demonstrated. Although vitamin K preparations, vitamin C, various hormones, and other drugs were used repeatedly, the only effective therapeutic measure was transfusion of blood or plasma.

The patient was receiving almost weekly infusions of plasma at the time of writing. This minimized but did not eliminate entirely the tendency to ecchymoses and deep hematomas. The joint aches, however, seemed to be well controlled. The last five hospitalizations occurred during periods when the plasma injections were not given with regularity.

*Physical Examination*—The patient was a well developed, robust appearing young woman. Examinations usually were within normal limits except for the ecchymotic areas and the deep hematomas from time to time. The hematomas usually were in the extremities, especially the lower extremities. No cardiac abnormalities were noted. The liver and spleen were not enlarged. No spider nevi were seen. A pelvic hematoma large enough to push the cervix to the interior and palpable three fingers above the symphysis pubis was present, as mentioned. In recent years no significant abnormalities were noted about the joint even when there was aching, none had limitation of motion. Neurological examination always was normal.

*Laboratory Findings*—Approximately 900 laboratory results were recorded. They can be summarized as follows:

The hemoglobin and erythrocyte values were normal except during the times of severe hemorrhages. The lowest levels resulting from epistaxis and laceration were 8.8 Gm hemo-

TABLE II CEPHALIN CHOLESTEROL FLOCCULATION

DATE	24 HR	48 HR
5/17/44	3+	4+
8/23/44	3+	3+
8/29/44	4+	4+
12/11/44	1+	2+
12/13/44	2+	3+
3/4/46	1+	2+
12/30/46	1+	1+
3/22/47	2+	2+

globin and 3,350,000 red blood cells, while during the period of uterine bleedings the value fell as low as 4.4 Gm hemoglobin and 1,460,000 red blood cells. White blood cells, differential and platelet estimations were normal. Results of bone marrow examination Mar 27, 1940, were not remarkable. Urinalyses showed no abnormalities if catheterized specimens were examined. Neither gross nor microscopic hematuria ever was proved. Blood calcium and vitamin C levels were normal. Plasma proteins including fibrinogen repeatedly were found within normal limits. Serologic tests for syphilis were negative. The patient's blood type was A. Liver function tests consistently showed only one aberration, namely a positive cephalin cholesterol flocculation test, as detailed in Table II. Serum bilirubin levels were normal except for one elevation noted in 1941—an icterus index of 35 which was checked three



days later and found to be 4. There were no symptoms of hepatitis noted at that time or recalled by the patient. She had received 1,000 cc of blood the preceding two days but no reaction was noted. Thymol turbidity, bromsulfalein urine and fecal urobilinogen urine coproporphyrin, cholesterol, cholesterol esters, alkaline phosphatase, hippuric acid excretion, galactose tolerance and stereobilin clearance tests were all within normal limits.

The usual tests of hemostasis give variable results. Sixty three per cent of forty nine clotting time determinations were prolonged. The clotting time was prolonged to 55 minutes twice once in 1938 and again in 1947. Approximately 40 per cent of thirteen retraction studies were abnormal. Bleeding times were prolonged in 43 per cent of forty six tests. Bleeding time recently was over 37 minutes, but usually when prolonged it was only mildly so. The cuff test was usually negative; only two tests out of thirteen were positive.

Prothrombin times were consistently elevated and in the same range over this period as illustrated by Table III.

TABLE III

DATE*	PROTHROMBIN TIMES (QUICK) (SEC)		CONCENTRATION OF PROTHROMBIN IN PLASMA (%)
	CONTROL	J. L.	
5/9/38	12	81	< 5
5/17/44	11	87	< 5
7/13/44	11.5	51.5	< 10
8/23/44	12	50	< 10
9/8/44	12	74	< 5
9/30/44	12	47	< 10
10/13/44	12.5	57	5
5/30/47	12	56.5	5
6/20/47	12.5	47.5	< 10
1/3/47	12	52.5	< 10

\* Selected from approximately 140 determinations on the basis of the control values which fall within Quick's 11 to 15 second range. This facilitates expression in per cent by Quick's chart.<sup>1</sup>

*Special Studies*—As Quick<sup>1</sup> has emphasized, prothrombin is estimated by a measure of its activity. The estimation is based upon certain assumptions. To prove that a delay in prothrombin time is due to prothrombin deficiency, other possible causes must be excluded. Several studies were made in the present case with this objective in mind.

TABLE IV CLOTTING TIME OF RECALCIFIED PLASMA<sup>14</sup>

	LOW SPEED CENTRIFUGATION (SEC)	HIGH SPEED CENTRIFUGATION (SEC)	VENOUS CLOTTING TIME (MIN)	PROTHROMBIN TIME
Normal plasma	113	141		
Hemophilic plasma	281	433	55	17.5/18 (control)
J. L.'s plasma	74	760	51	39.7/44.4 (control)

*1 Effect of Platelets* In Quick's test the thromboplastic factor is assumed to be eliminated by the addition of an excess of thromboplastin. Nevertheless, it was thought of interest to examine the effect of platelets in the present case. This was done by comparing the recalcified clotting times of low and high speed centrifugalized plasma as outlined by Quick<sup>14</sup>. The test was carried out with the results outlined in Table IV. These results reveal a behavior quite unlike that in hemophilia.

By Miss Elaine Daughenbaugh, Instructor, School of Medical Technology, University of Minnesota.

2 *Effect of Purified Prothrombin in vitro* This study was made in order to exclude the possibility of an interference with the conversion of prothrombin. This might be caused by a factor such as excessive trypsin inhibitor<sup>16</sup> or, as recently suggested,<sup>17</sup> by a lack of plasma accelerator factor (Ae globulin). Purified prothrombin\* was prepared by the method of Seegers and associates.<sup>18</sup> A comparison was made of the effect of progressive concentrations of this material on the prothrombin times of old plasma, dicumarolized plasma, and plasma from our patient.

TABLE V PROTHROMBIN TIME RESPONSE TO VARIOUS CONCENTRATIONS OF PURIFIED PROTHROMBIN\*

CONCENTRATION OF ADDED PURIFIED PROTHROMBIN (MG %)	OLD PLASMA (SEC) (Control 15.5)	DICUMAROLIZED PLASMA (Control 16)	J L'S PLASMA (Control 16)
0	49	30	53
1	46.5	30	42
2.5	39	29	31.5
5	33.5	28	27.5
10	28.5	25.5	21.5
15	24.5	20.5	19.5
20	22.5	23.5	16.5
100	15	15.5	13.5

\*Supplied through the kindness of Dr Walter H Seegers Wayne University Medical School Detroit Mich

TABLE VI CLOTTING TIMES OF DECALCIFIED PLASMA TREATED WITH DECRYFASING DILUTIONS OF THROMBIN

THROMBIN DILUTIONS	CLOTTING TIME (SEC)	
	NORMAL	J L
Full strength*	3.5	3
1:2	4	3.5
1:5	4.5	5
1:10	13.5	10
1:20	20	17
1:40	47.5	29.5
Prothrombin time	15	53.5

\*Diluted solution of thrombin topical Parke Davis & Company Detroit, Mich

Purified prothrombin was added to portions of the respective plasmas to make 100 mg per cent concentrations. Then a series of dilutions with the proper untreated plasmas produced several concentrations of the added prothrombin. Quick prothrombin estimations were made on the resulting prothrombin solutions. The results are summarized in Table V. Comparison shows that our patient's plasma responded to added prothrombin as well as and in a manner similar to plasma made hypoprothrombinemic by oxidation or dicumarolization. The defect, therefore, did not seem to be an interference with prothrombin conversion.

3 *Thrombin Clotting Study* Defects in the second phase of coagulation can cause a prolongation of the prothrombin time. These may be anticoagulants.

\*Supplied by Dr Walter H Seegers

which inhibit the effect of thrombin, or the fibrinogen may be qualitatively defective or markedly diminished as in afibrinogenemia.<sup>19</sup> As noted the quantity of fibrinogen in our patient was normal. Anticoagulant effect and/or fibrinogen defect may be detected by clotting decalcified plasma with increasingly dilute solutions of thrombin.<sup>1</sup> Table VI, summarizing the results of such a study reveals no evidence of an anticoagulant, or a defect in fibrinogen. Such abnormalities were thus not the cause of the delayed prothrombin time in our patient.

4 *Another Test for Anticoagulant* If plasma presumed to be hypoprotrombinemic, contains an anticoagulant it should prolong the prothrombin time of a control beyond that expected by dilution. However, several tests showed that J. L.'s plasma did not prolong the time of the control as much as did the addition of saline. A typical experiment resulted as follows: control plasma was 12 sec., control plus an equal amount of saline gave 16.5 sec., control plus J. L.'s plasma in equal amounts was only 13.5 sec. Again an anticoagulant could not be demonstrated in the blood.

5 *Two Stage Prothrombin Determination* To help determine whether a retarded conversion rate caused the delayed prothrombin time the procedure was done in two stages as advocated by Warner, Binkhous, and Smith.<sup>19</sup> Prothrombin times (Quick) on control and J. L.'s plasmas were 18 and 60 respectively. The plasmas were defibrinated by the addition of thrombin solution. Part of the defibrinated control plasma was diluted 1:3. One tenth cubic centimeter of each of the three plasma samples was incubated for seventy seconds with  $\text{CaCl}_2$  (0.1 cc of 0.25M) and thromboplastin (0.1 cc) solutions. Fibrinogen solution (0.1 cc) (Cohn's Human Fraction I used as source) was added to each and the clotting times were noted. These were 16.5, 41.5 and 58 seconds for control, diluted control, and J. L.'s plasmas, respectively. These results would indicate that our patient's deficiency was lack of prothrombin and not a delayed rate of convertibility.

*Special Examinations of Plasma Proteins*—(1) Electrophoretic studies of J. L.'s plasma\* in 1945 revealed no significant abnormalities. (2) A cryoglobulin was found in abnormal amount. The characteristics of this protein are reported elsewhere.<sup>20</sup> It may be mentioned here however that this cryoglobulin was of unusual interest in that unlike others which have been studied it contained a carbohydrate fraction.<sup>20</sup> (3) The quality of the fibrinogen was found to be normal by thrombin clotting as detailed previously. In addition however the effect of addition of fibrinogen was tested. Bovine fibrinogen,† found to be normally active by thrombin clotting was dissolved in control and J. L.'s plasmas to make a concentration of 0.5 per cent. The prothrombin times were as follows: control 15 sec., control plus fibrinogen 15 sec., J. L. 53.5 sec., J. L. plus fibrinogen, 48 sec. This apparent 5.5 sec. decrease in the prothrombin time was not considered significant.

*Component Assay*—Quick<sup>4</sup> has postulated that prothrombin is a complex consisting of calcium, component A, which is an oxidation labile factor and component B, which is removed by decarboxylation *in vivo* and by aluminum

\* By Dr. H. L. Taylor, Laboratory of Physiological Hygiene, University of Minnesota.  
† Supplied by Dr. E. C. Loomis of Parke, Davis & Company, Detroit, Mich.

hydroxide in vitro. More recently Quick<sup>6</sup> speaks of component B as the "conventional prothrombin" and describes a new component A, using the term "labile factor" for the old component A. Seegers and co-workers,<sup>13</sup> however, affirm that prothrombin is composed of but a single component. Deficiency of component B was found in the cases of Lewis and Bennett<sup>11</sup> and of Quick.<sup>12</sup> Owien<sup>13</sup> has described a case which may represent a deficiency in the labile factor.<sup>6</sup> Although at present the exact status of the components is not clear cut, we attempted to determine the type of component deficiency in our patient. Oxalated plasmas stored approximately one month were used as a source of plasma deficient in the labile factor. Plasma from dicumarolized patients served as a source for component B deficient plasma. The results of the experiments are recorded in Table VII. Since old plasma was more effective than dicumarolized plasma in reducing J. L.'s prothrombin time, it appears that the more important deficiency was of component B. However, the results are difficult to interpret. They may indicate that there was also some deficiency in the labile factor since, in four out of five experiments, stored plasma was not so effective as control plasma in restoring the prothrombin time of J. L.'s plasma. In three of these four experiments, however, dicumarolized plasma plus stored plasma did not result in a normal prothrombin time. Therefore, the possibility exists that there was a deficiency of some other unknown factor.

TABLE VII PROTHROMBIN COMPONENT ASSAY

	PROTHROMBIN CLOTTING TIME (SEC)				
	3/25/47	6/12/47	6/19/47	7/2/47	7/3/47
Control	14.5	14.5	15	14	12
Stored plasma	34	67	61	54	6.5
Dicumarolized plasma	31.5	40	43	54.5	33.5
J. L.'s plasma	37	39.5	53.5	53.5	5.5
J. L. + control	17	15.5	16.5	16.5	13.5
J. L. + stored plasma	21.3	17.5	16.5	21.5	13.5
J. L. + dicumarolized plasma	24	26.5	20	36	29
Control + saline				18	16.5
Control + stored plasma		15.5	15	17	16
Control + dicumarolized plasma		18	16	17	14
Dicumarolized plasma + stored plasma		18	14.5	21	16

*Response to Vitamin K*—Throughout the patient's course vitamin K and various synthetic vitamin K products in usual doses were tried without effect. The first massive dosage of synthetic vitamin K was given in 1944. Over a three day period the patient received intravenously a total of 800 mg. of hydroxide.\* This was associated with a rise in prothrombin time from 70.5 to 102 seconds, the controls had a time of 17 seconds. Recently, six days after a plasma infusion, 72 mg. of menadione bisulfite were administered intravenously. The prothrombin time rose from 34 to 51.5 seconds (controls, 12 and 13 seconds) over a twenty-four hour period. This demonstrates that the patient continued to be vitamin K resistant.

\*This study was made by Dr. Rudolf Marshall at that time a Fellow in Medicine, University of Minnesota Hospital.

*Response to Plasma*—Evidence of consistent therapeutic response to blood or plasma transfusions was recorded throughout the entire course of observation. The prothrombin times regularly decreased following such infusions. A few of the more recent examples are summarized in Table VIII. It will be noted that plasmas ten, fourteen, and thirty days old were about as effective as those only three days old. This is confirmatory evidence that the patient's main deficiency was not of the labile factor. Also this observation is of considerable practical importance since stored plasma is readily available in blood banks whereas there may be some difficulty in obtaining immediately fresh plasma or blood. Pooled dried plasma, because it has been a source of the hepatitis virus was not given a trial in our patient.

TABLE VIII PROTHROMBIN TIME RESPONSE TO PLASMA OF VARIOUS AGES AND QUANTITIES

DATE	NORMAL	J. I.'S PROTHROMBIN TIME BEFORE PLASMA	PLASMA (CC)	AGE OF PLASMA (DAYS)	J. I.'S PROTHROMBIN TIME AFTER PLASMA
1 <sup>9</sup> /30/46	13	35	250	30	21.5
4/ 4/47	13.5	55	180	14	31.5
4/ 1/47	14.5	45	250	10	21.5
5/10/47	12	56.5	250	3	26
6/23/47	12.5	47.5	230	3	24
7/ 3/47	12	52.5	100	3	34
	50% solution* 16.5	34	100	3	28.5
		28.5	50	3	29
7/14/47	14.5	59	100	3	45.5
	50% solution* 22	45.5	100	3	36
	30% solution* 35	36	100	3	33.5
	20% solution* 50	33.5	150	3	30
	10% solution* 145				

\*Dilution of plasma with physiological saline

The bleeding and clotting times seem to respond favorably with the plasma injections. For instance, on June 19, 1947, the venous clotting time fell from 39 minutes to 15 minutes after 250 cc of plasma. The bleeding time (Duke) decreased from 9 minutes 45 seconds to 6 minutes 30 seconds. The prothrombin time diminished from 53.5 to 24 seconds (control, 15 seconds). On June 3, 1947, the ear lobe puncture continued to bleed until the plasma infusion was completed.

*Capillary Examination*—Microscopic observation of the capillaries of the nailbed was made\*. Many so called abnormal formations were observed but not in larger proportion than often seen in normal persons. Blood flow was normal in rate and appearance. Traumatization is suggested by Macfarlane<sup>1</sup> showed the injured capillary loop to disappear in normal fashion. Our patient's capillaries, therefore, were normal insofar as this type of examination can indicate.

*Studies on Available Family Members*—These studies are summarized in Table IX. Only the mother and the patient noted easy bruising. However, all members except the father showed prolongation of the prothrombin time. Although detailed assay studies were not done, addition of control plasma to plasmas from three of the family resulted in the same type of response as shown with the patient's plasma. Presumably they all had the same type of defect but in different degrees. The sister had a positive cuff test but no history of

TABLE IX PROTHROMBIN AND OTHER DATA IN THE IMMEDIATE MEMBERS OF THE FAMILY OF J. L.

	AGE (YR.)	DATE	CONTROL (SFC)	50% SALINE SOLUTION OF CONTROL (SEC.)	PROTHROM- BIN TIME (SEC.)	50% SOLUTION WITH CONTROL (SFC)	50% SALINE SOLUTION (SFC)	BLEEDING TIME	CLOTTING TIME	CLOT REFRACT TION	CUFF TEST	EASY BRUISING
Mother	15	7/ 8/47	12	17.5	16	14	22	Normal	Normal	Normal	Positive	Yes
Father	55	7/ 8/47	12	17.5	12		19	Normal	Normal	Normal	Negative	No
Sister	26	7/14/47	14.5	21.5	16	15.5	26	Normal	Normal	Normal	Positive	No
Brother T	22			Not available for study								
Patient	20	7/ 3/47	12	16.5	52.5	13.5		6/12/47 Normal	Prolonged	Normal	Negative	Yes
Brother R	17	7/ 2/47	13	18	17			6/19/47 Normal	Normal	Normal	Negative	No
Brother D	13	7/14/47	14.5	21.5	18	15.5	32	6/11/47 Normal	Normal	Normal	Negative	No

bruising. However, it may be of significance that her 2 year old daughter was said to bruise easily, so far she had had no episodes of bleeding. These examinations certainly establish the familial nature of the disease in our patient.

#### DISCUSSION

The history and findings of a patient with idiopathic familial hypoprothrombinemia have been presented. The patient was followed in detail over a ten year period. There was a variety of studies and therapeutic attempts in order to ascertain the exact pathologic mechanism. All of the known factors other than hypoprothrombinemia which might give rise to a delay in prothrombin time were presumably excluded by the experiments detailed. There was an evident lack of the prothrombin factor which is decreased by Dicumarol (component B). There was no response to vitamin K while plasma of various ages was consistently effective. The regular occurrence of a positive cephalin flocculation test, albeit in varying intensity together with the presence of a cryoglobulin in the patient's serum tends to support the concept of a primary disturbance of protein synthesis in the liver.

The most important result of our studies was the evidence of familial incidence of the disorder. Those afflicted seemed to have the same type of deficiency. This is the fifth reported instance of familial hypoprothrombinemia. Actually the incidence is probably higher than this would indicate. Reference to Table I shows that in most cases there was a familial hemorrhagic tendency, but prothrombin estimations were not always made. Moreover, just a statement of a negative family history is not sufficient since several persons with no hemorrhagic manifestations have been found to be mildly hypoprothrombinemic. This was true in the family of J. L. Whenever the diagnosis of idiopathic hypoprothrombinemia is entertained, prothrombin determinations should be made on all available family members to help clarify the diagnosis.

#### SUMMARY AND CONCLUSIONS

A case of idiopathic hypoprothrombinemia followed in detail for a decade has been presented. The hemorrhagic disease in this case was characterized by epistaxis, subcutaneous hematomas, hemorrhages in proximity to various joints, menorrhagia and metrorrhagia, the latter being so severe as to require hysterectomy.

The familial character of the disease has been established in this instance. The major deficiency was of the Quick B component that affected by Dicumarol, yet vitamin K in large amounts was ineffective in shortening the prothrombin time. Purified prothrombin and human plasma, either old or fresh, were the only materials effective in this regard in vitro; the former was not used in vivo, while the latter was consistently effective in controlling the hemorrhagic tendency.

Positive cephalin flocculation and the presence of a cryoglobulin suggest that there may have been a primary disturbance of protein synthesis in the liver.

It is a pleasure to acknowledge the helpful advice and criticisms of Dr. Armand Quick during the course of this study.

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# OBSERVATIONS ON ALCOHOLIC FATTY LIVER THE USE OF INTERVAL NEEDLE BIOPSY AND LIVER FUNCTION TESTS

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## INTRODUCTION

**D**URING the past eight years, needle (trocar) biopsy of the liver has become a recognized procedure in many clinics. Its value in clinical investigation and in the diagnosis of obscure cases of liver disease is well established.

This is a report of observations on seven cases of so called alcoholic liver. Biopsies give evidence of rapid disappearance of fat following therapy in five of these cases. An attempt will be made to correlate the more important liver function tests with these changes along with the clinical features of the cases and the response to therapy.

Hoffbauer<sup>1</sup> recently gave a summary of the different techniques and of the indications for and dangers and advantages of needle biopsy. In a series of 1,200 cases, Sherlock<sup>2</sup> reported a mortality of 0.67 per cent. Hemorrhage is by far the most common cause of death. Other disadvantages include the failure always to secure an adequate specimen and the possibility that one shred of tissue may not represent accurately the picture of the rest of the liver. However the needle biopsy usually does offer a more representative picture than the ordinary surgical biopsy because the latter penetrates only a small distance through the capsule.

The pathogenesis of fat infiltration in the liver has been studied extensively in animals in recent years. By the use of serial needle biopsies the pathogenesis of the so called fatty liver in man should now be amenable to investigation.

Experimentally, fatty livers may be divided into two types<sup>4</sup> those produced by an increase in the rate at which fat is supplied to the liver and those produced by a decrease in the rate at which the liver is able to dispose of fat. In the first category belongs the fatty liver produced by starvation, high fat diet and stimulation with the ketogenic fraction of the anterior lobe of the pituitary.<sup>5</sup> The second group includes fatty livers resulting from poisoning with carbon tetrachloride or phosphorus, depancreatization plus insulin (dogs) and choline deficiency combined with a low protein diet. Some investigators<sup>6</sup> believe that cirrhosis in man as in certain experimental animals<sup>7</sup> may be the end result of extensive fat infiltration.

Incipient cirrhosis in alcoholics often is characterized by fatty infiltration and enlargement of the liver.<sup>8</sup> It is in such cases (as shown below) that spontaneous remissions occur when alcohol is withdrawn and a satisfactory dietary regime is established. Treatment does not cause disappearance of the fibrosis but presumably does aid in liver cell recovery and regeneration.

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In clinical therapeutic studies with fortified diets of various types, it has been noted that the prognosis in cirrhosis seems related to liver size.<sup>9, 10</sup> Few subjects with small livers did well with any therapy, whereas those with large, easily palpable organs improved, presumably because of resorption of liver fat. Evidence is presented here that this is indeed the case.

#### MATERIAL AND METHODS

During the year ending in June, 1947, a total of fifty six needle liver biopsies on twenty eight patients has been attempted at the Salt Lake Veterans' Hospital and the Salt Lake County General Hospital. There were twenty two failures (due to insufficient tissue for making a microscopic diagnosis). The Roth Turkel needle was used in all instances and the technique as described by Davis and co workers<sup>3</sup> was followed. When the liver was easily palpable, it was usually approached from below the right costal margin anteriorly. An intercostal approach in the anterior or posterior axillary line was used when the liver was not palpable.

Only the liver function tests used routinely in these selected cases are recorded in Tables I and II. These were done by generally recognized methods.<sup>11, 14</sup>

Emphasis will be placed here on a selected group of seven white, male alcoholics with fatty livers. In Tables I and II a summary of the important clinical features, liver function tests, histologic observations, and treatment of these subjects is given.

Two needle biopsies were performed on each of the seven patients, the first soon after admission and the second after an interval of from three weeks to three and one half months. The first five cases summarized in the tables had a favorable outcome. Patient B N (Case 6) was committed to the state mental hospital where he expired within a month. Autopsy was not performed. Case 7 is included to contrast the course with that of the first five cases and also because the immediate cause of death was hemorrhage following a needle biopsy. One other complication occurred in the total series—a right pneumothorax. Recovery was uneventful.

#### CLINICAL OBSERVATIONS

All of these patients gave evidence of prolonged and severe alcoholism on admission to the hospital. The salient symptoms and signs for each are listed in Table I. They were the usual findings in varying degrees of decompensated cirrhosis and nutritional deficiency.

#### LABORATORY FINDINGS

The laboratory data before treatment indicated mild to moderate hepatic insufficiency. The cephalin-cholesterol flocculation test was initially positive in three of the seven cases. Of the five cases in which the thymol turbidity was measured on admission, the findings were abnormal in only two. Moderate to marked hypoalbuminemia was present in five of the seven subjects. There was bromsulfalein retention in all the patients and four had an initial mild anemia of the macrocytic type.

#### HISTOLOGIC OBSERVATIONS

The needle liver biopsy, done on all patients at the time of admission, showed moderate to marked diffuse fatty infiltration and from normal to moderate increase in fibrous tissue in the periportal areas. Inflammatory cells, mostly lymphocytes, were present in varying numbers at the triads. Scattered areas of degeneration of liver cord cells were observed in three patients on admission.

TABLE I CLINICAL DATA BEFORE AND AFTER TREATMENT

CASE	PATIENT	TIME INTERVAL (Wks.)	JAUNDICE	LIVER SIZE*	ASCITES	ANKLE EDEMA	OTHER MANIFESTATIONS	THERAPY
1	H K	4	++	7	+	++	Peripheral neuropathy delirium tremens	Diet† and B complex vitamins after 1000 cc Amigen and 1000 cc 10 per cent glucose daily for 10 days
			0	1	0	0	Asymptomatic gained 30 pounds	
2	W S	6	+++	10	++	0	Acutely ill, mentally obtunded, spider nevi	300 cc Parenamine in 1 liter 5 per cent glucose daily for 1 week plus multivitamins, then diet plus 300 cc Parenamine orally daily
			+-	6	0	0	Much improved, gained 18 pounds	
3	A G	4	0	4	+	+	Tremor of hands not acutely ill	House diet no other vitamins or other supplemental therapy
			0	1	0	0	Asymptomatic lost 10 pounds	
4	W O	4	+	4	0	0	Acutely ill	Diet† only no supplementary vitamins
			0	1	0	0	Asymptomatic, no change in weight	
5	H P	14	0	3	0	0	Delirium tremens, peripheral neuropathy	In hospital (8 weeks), diet† B complex vitamins plus 90 cc Protolysate per day
			0	Not palpable	0	0	Asymptomatic except neuropathy unchanged	Out patient (6 weeks) no special diet, no alcohol
6	B N	3	0	8	++	0	Korsakow's psychosis	Diet† did not eat well choline 4 Gm per day
			0	8	+	0	Unimproved, died in another hospital	
7	F W	13	0	4	0	0	None	Diet† and B complex vitamins. Improved in hospital 2 weeks, out of hospital 10 weeks did not abstain from alcohol, died following liver puncture
			++++	9	+++	+	(Third admission) Stuporous, critically ill	

\*Liver size is in centimeters below the right costal margin in the midclavicular line  
†Diet was composed of protein 150 Gm carbohydrate 300 Gm fat 10 Gm

Treatment consisted essentially of rest, withdrawal of alcohol a high protein, high carbohydrate, and low fat diet, and B complex vitamins

## OBSERVATIONS AFTER TREATMENT

Results of treatment can be summarized as follows

- 1 Rapid clinical improvement (first five cases) such as gain in appetite strength, and weight diminution in liver size, edema and ascites and clearing of icterus in the jaundiced patients
- 2 Consistent and significant increase in serum albumin
- 3 Return of hemoglobin to normal

TABLE II

			LABORATORY DATA								HISTOLOGIC DATA		
CASE	PATIENT	TIME INTERVAL (WK)	THYMOL TURBIDITY (UNIT)	CEPHALIN FLOCCULATION	SERUM ALBUMIN (GM /100 CC)	SERUM GLOBULIN (GM /100 CC)	BSP RETENTION (%)	VAN DEN BERGH (1 SEC)	TOTAL VAN DEN BERGH	HEMOGLOBIN (GM /100 ML)	LYMPHOCYTIC INFILTRATION	FAT	BIOPSY CHANGES
1	H K	4	—	3+	3.6	2.6	29	16	12.0	12.0	+	3+	2+
			6.0	3+	4.2	3.5	6	6	16	13.5	+	1+	2-
2	W S	6	7.0	4+	3.0	3.5	—	25	27.0	12.5	+	4+	2+
			6.5	Neg	4.1	4.2	15	3	4.1	16.0	4+	1+	3
3	A G	4	3.0	Neg	5.3	2.6	21	3	1.2	15.0	+	4+	1-
			5.0	Neg	—	—	15	1	1.0	15.0	+	1+	0
4	W O	4	16.5	4+	3.7	1.9	18	8	8.0	12.3	+	2+	1
			9.0	Neg	4.8	2.0	8	3	1.2	15.0	2+	0	1
5	H P	14	2.5	Neg	4.0	3.3	23	5	1.4	15.0	2+	3+	2-
			2.5	Neg	4.1	3.3	17	3	9	17.0	3+	0	2+
6	B N	3	—	Neg	4.1	2.8	—	4	9	11.0	2+	3+	2-
			—	—	—	—	—	—	—	—	+	2+	2+
7	F W	13	4.0	—	5.4	2.2	40	8	2.4	18	+	1+	2+
			8.0	3+	2.5	3.5	—	8.4	35.0	14.5	Polys* 4+	1+	1+ Wide spread necrosis

— Tests were not done

1+ Approximately amount seen in Fig 4 4+ illustrated by Fig 3

\*Polymorphonuclears

4 Reversion of the cephalin-cholesterol flocculation to negative in these cases in which it was positive

5 In contrast, only in one case out of the five in which two tests were performed was significant improvement in the thymol turbidity observed

6 Decrease in the quantity of bilirubin in the serum in the jaundiced patients with corresponding change in the bromsulphalein test. The latter returned to normal in two instances

7 Histologically, there was a consistent and marked diminution in fat in the first five subjects without marked change in periportal fibrosis and signs of inflammation. Many cells showed double nuclei. This was interpreted as evidence of regeneration

#### COMMENT

It was pointed out by Hoffbauer, Evans, and Watson<sup>15</sup> that it is not always possible to correlate closely liver function tests and anatomic findings. The same impression is gained here in cases of fatty liver. The bromsulphalein retention test and the serum albumin level seemed to be the most consistent in reflecting the clinical and histologic status before and after treatment.

It may be noted (see Table II) that the Hanger test may be negative when the liver is fatty. Thymol turbidity was variable and not markedly elevated. Similar results for these tests in cirrhosis have been noted before.<sup>16, 17</sup>



Fig 1—Case 9. Specimen taken on admission showing marked fatty infiltration and slight periportal fibrosis and lymphocytic infiltration (low power hematoxylin and eosin)



Fig 2—Case 9. Specimen taken six weeks later showing marked diminution of fat with up to a fourfold increase in fibroblasts and lymphocytes (van Gieson)

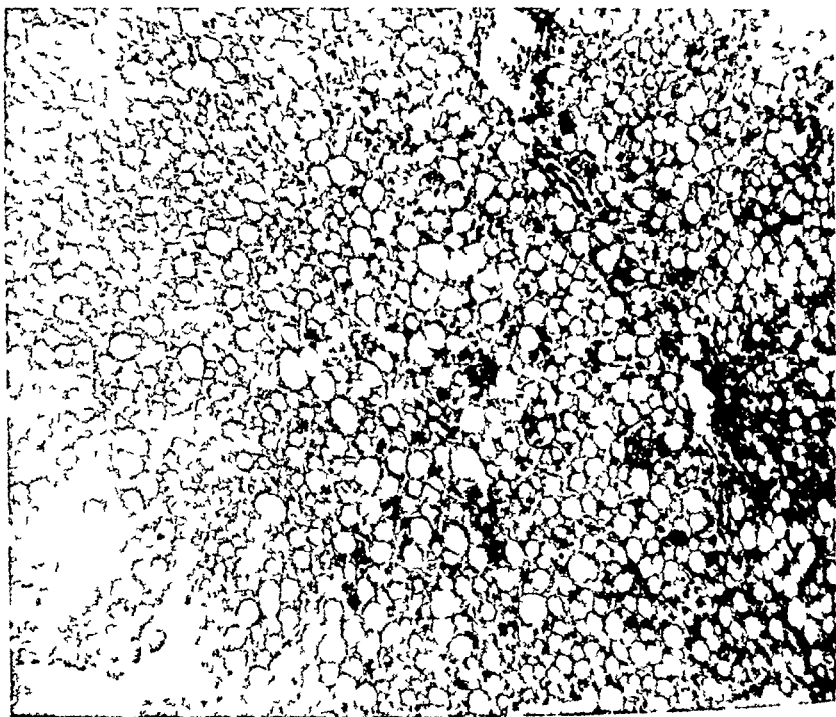


Fig 3—Case 3 Specimen taken on admission showing marked fatty infiltration (hematoxylin and eosin)

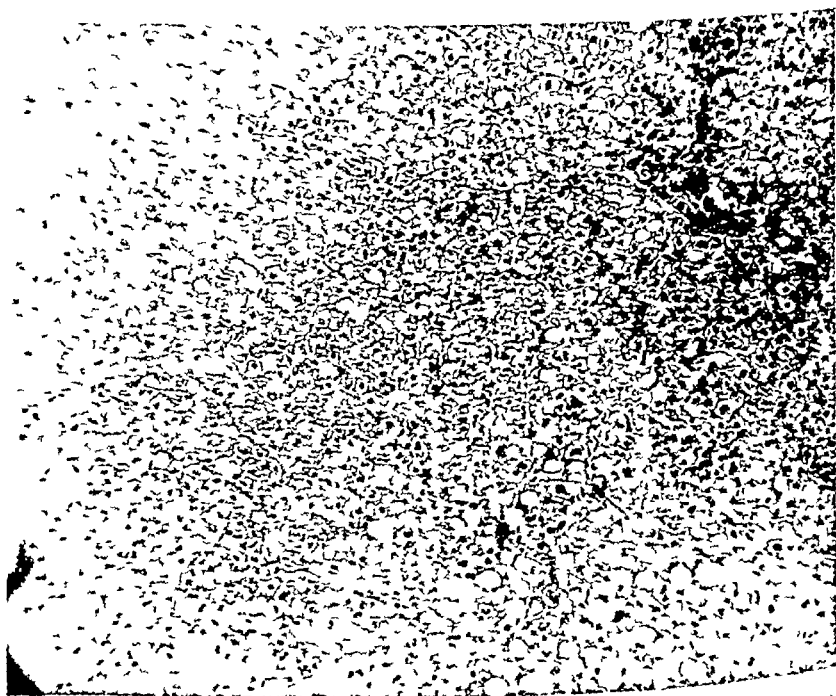


Fig 4—Case 3 Specimen taken four months later demonstrating marked decrease in fatty infiltration (hematoxylin and eosin)

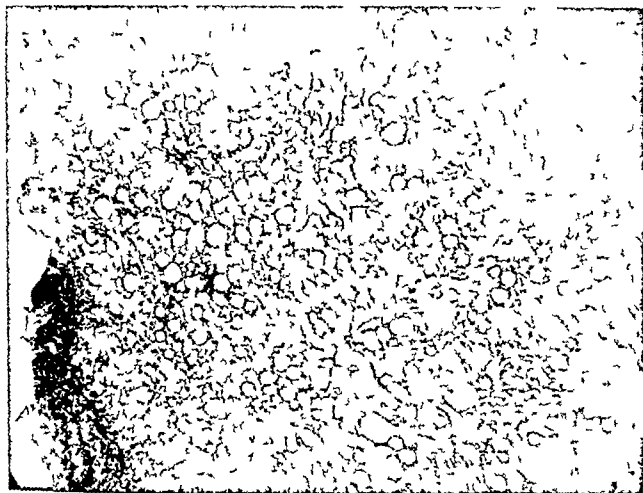


Fig. 5.—Case 7. Specimen taken thirteen weeks before death, showing moderate increase in fat and early cirrhosis (van Gieson).



Fig. 6.—Specimen taken on day of death (Case 7) showing cellular necrosis and dense polymorphonuclear infiltration (van Gieson).

The finding of principal interest was the anatomic evidence of rapid diminution of fat in the first five cases. It seemed remarkable that the liver could dispose of what appeared to be an enormous amount of fat in the short time interval of four weeks (in three cases). (See Figs 1 to 4) Presumably the processes concerned in lipid transport from the liver become effective soon after the initiation of therapy. The type of treatment seemed to make no particular difference as judged by the clinical result, function tests, and biopsy changes.

Two patients received amino acids parenterally as well as B complex vitamins and glucose intravenously until their appetites returned. Then they were given a diet of protein, 150 Gm, carbohydrate, 300 Gm, and fat, 70 grams. Patient A G (Case 3) received the house diet only (no vitamins or supplementary nourishment) and yet made a rapid recovery as judged clinically and by laboratory tests. In contrast to this, Patient B N, who was psychotic and ate poorly but received 4 Gm choline per day, showed no appreciable change in liver fat over a three-week period.

In the course of this study, one fatality occurred as a consequence of liver biopsy. Patient F W (Case 7, Tables I and II) expired three hours following the second (interval) liver puncture. This admission was his third within a year for liver disease. The patient was critically ill, with the signs and symptoms of acute liver failure. At autopsy death was shown to be due to hemorrhage from the liver biopsy wound and there was widespread necrosis and hepatitis superimposed on a fatty cirrhosis (Figs 5 and 6).

Liver puncture in this case was performed in the face of two contraindications: a prothrombin time of 50 per cent, not affected by vitamin K, and a patient too ill to cooperate. Death occurred without warning by any signs or symptoms of hemorrhage.

#### DISCUSSION

It appears that a good clinical result is obtainable in cases of fatty liver as long as the patient can eat, rest, and abstain from alcohol. It seems doubtful that the addition of lipotropic agents would have altered the course in the cases discussed since the rate of disappearance of abnormal amounts of lipid, in at least three of the cases, would seem to be difficult to improve upon.

The clinical syndrome of an alcoholic who enters the hospital acutely ill with jaundice, edema, ascites, and hepatomegaly is usually classified as cirrhosis of the liver. Actually all these symptoms or signs of hepatic decompensation can be produced by marked fatty infiltration and cellular damage with very little actual cirrhosis. Death may occur suddenly and at autopsy little or no trace of cirrhosis may be found.<sup>21, 22</sup> Or, as reported here in the first five cases, rapid, almost complete recovery may take place. Fatty changes and the accompanying signs of hepatic insufficiency may closely simulate those of cirrhosis yet the process is usually rapidly reversible with appropriate dietary therapy plus abstinence from alcohol.

It may be pointed out that treatment is the same regardless of the degree of cirrhosis present. The need for obtaining a picture of the histologic appearance of the liver may therefore be questioned. However, the initial pathologic



finding of only fat infiltration and the later demonstration of a relatively normal liver can be a powerful stimulus in indicating the value of an attempt at rehabilitation of the patient. The usual alcoholic relapse on discharge from the hospital might be averted if the patient and his physician had a clear picture of the changes in the liver associated with therapy and abstinence from alcohol and poor dietary habits. Resumption of former habits almost certainly would result in another episode of liver disease, whereas with abstinence, a lifetime free from liver disease can at least be hoped for.

#### SUMMARY AND CONCLUSIONS

Experience with fifty six attempts at needle puncture biopsy of the liver (including one pneumothorax and a death from hemorrhage) is reported.

Follow up biopsies performed in seven patients with alcoholic liver at intervals of from three weeks to three and one half months are presented, as well as simultaneous liver function studies and clinical findings.

Clinical improvement (coinciding with rapid diminution in the quantity of liver fat) was noted in five patients, and similar improvement was observed in liver function tests. In patients who could eat an adequate diet the use of supplementary lipotropic agents seemed unnecessary.

Fatty liver with a mild underlying cirrhosis may exhibit all the clinical signs of an advanced portal cirrhosis and is usually diagnosed as such. Differentiation is important in prognosis.

It is believed that if the procedure is carried out by the fewest possible operators and if due attention is given to the contraindications for the procedure liver puncture is justified in selected cases for diagnosis and prognosis. This does not imply that it should be a routine method in the clinical diagnosis of liver disease.

Serial biopsies in early cirrhosis over a period of years would add much in elucidating the natural history of the disease.

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# THE THYMOL TURBIDITY TEST IN VARIOUS DISEASES

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THE thymol turbidity test was first described by MacLagan<sup>1, 2</sup> in 1944 as an index of liver function. Since then many papers have appeared in the literature concerning its value. The exact mechanism of the test has not as yet been completely determined. The turbidity is due to the formation of a complex consisting of a globulin, phospholipid, cholesterol and thymol. MacLagan believed that the globulin was gamma globulin and that the thymol turbidity and cephalin flocculation tests had a similar mechanism. Recant and co workers<sup>3</sup> using electrophoretic methods, were able to show that gamma globulin was not involved in the mechanism of the thymol turbidity test while the cephalin flocculation test depended on the presence of gamma globulin. Recently Cohen and Thompson<sup>4</sup> presented evidence that the protein in the complex of the positive thymol turbidity test was beta globulin. Clinically investigators<sup>5, 6</sup> have felt that the basic mechanisms of the thymol turbidity test and the cephalin flocculation test were different. Kunkel and Hoagland<sup>7</sup> have offered experimental evidence to show that the development of the turbidity depends on both lipids and gamma globulins in the serum. However the lipid protein complex migrates in the beta globulin fraction of the serum.

The thymol turbidity test has many advantages over other tests of liver function in current use that is stability of reagents, quantitative method of determination, short interval of time needed for performance and apparent sensitivity. It was therefore decided to evaluate this procedure by testing sera from patients with liver disease as well as various other diseases.

## MATERIALS AND METHODS

The technique used for the performance of the thymol turbidity test was the modification suggested by Shank and Hoagland<sup>8</sup>. Sera showing 5 or more units were classed as abnormal. This was based on our own observations of the test and on the investigations of Hoagland and Shank. These workers used 47 units as the upper limit of normal. A cephalin flocculation test was performed on each serum at the same time that the thymol turbidity determination was done. The cephalin flocculation test was read at the end of forty eight hours and was called positive if it read 3 plus or 4 plus. In most instances total protein and formal gel determinations were also carried out. Bromsulphalein tests were done when indicated.

Although many of the patients tested were thought to have liver dysfunction a large number of tests were made on patients in whom liver disease was not thought to be present. Many patients were tested repeatedly.

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## RESULTS

Tests were carried out one or more times on 567 persons. Seventy-four of these were regarded as normal controls. The results are presented in Table I, in which an attempt has been made to classify the cases. It will be noted that a positive result was obtained in 295, or 52 per cent of all persons tested.

Certain comments may be made regarding the results in the different groups.

TABLE I

GROUP	NUMBER OF CASES	NUMBER T T +	PER CENT T T +	POSITIVE THYMOL TURBIDITY TESTS	
				AVERAGE NUMBER OF UNITS	RANGE
Infectious hepatitis	36	34	94.5	23	5-50
Cirrhosis of the liver	48	46	96	20	5-49
Obstructive jaundice	12	5	42.5	17	6½-38
Diseases of the gall bladder	11	6	54.5	8	5-20
Weil's disease	4	4	100	17.4	8-34
Diseases with widespread liver destruction	11	8	73	14	6-33½
Neurosyphilis with fever therapy	9	9	100	19	8-28
Neurosyphilis without fever therapy	9	2	22.2	10.3	6½-14
Acute lymphogranuloma venereum	21	20	95.3	13.6	7-30
Acute and chronic rheumatoid arthritis	17	14	82.4	10	5-19
Acute rheumatic fever	11	6	54.5	6.7	5-9
Congestive heart failure	56	27	48	8.4	5-20
Heart disease without failure	19	9	47	8.4	7-15½
Chronic lung disease	28	15	53.5	11	5-20
Acute infectious diseases	67	25	37.3	9	5-23
Neoplastic disease	15	7	46.5	9.5	5-16
Diabetes mellitus	19	6	31.6	8.6	5½-10½
Ulcers and gastrointestinal hemorrhage	18	3	16.7	8	6½-11½
Thyrotoxicosis	7	4	57	9.8	5½-14
Nutritional disease	10	6	60	8	5-16½
Chronic ulcerative colitis	3	2	67	9	6-17
Amebiasis	4	0	-	-	-
Hemolytic crises	5	4	80	8	6-11½
Chronic alcoholism	4	0	-	-	-
Miscellaneous	49	28	57	7.5	6½-9½
Controls	74	6	8	-	-
Total	567	295	52	-	-

*Infectious Hepatitis*—Series of thirty-six cases of infectious hepatitis were tested, four of these were probably cases of homologous serum jaundice. All but two of the thirty-six had a positive test, the average number of units being 23. One of the remaining cases had a negative test initially but one week later the result was positive. In the remaining case, which was typical of infectious hepatitis clinically, the thymol turbidity test remained negative throughout the hospital course. These results agree closely with those quoted in the literature.

*Cirrhosis of the Liver*—There were forty-eight subjects in this series and forty-six of them gave a positive thymol turbidity test. The average number of units was 20. A study of our cases revealed that the thymol turbidity test showed no essential difference between the cases of cirrhosis which occurred in alcoholics and those which occurred in nonalcoholics. Chronic hepatitis following infectious hepatitis cannot be separated from cirrhosis by the thymol turbidity test.

Of the two patients with cirrhosis with negative thymol turbidity tests on admission, one turned positive a month later while the other remained negative throughout the observation period of two weeks

*Obstructive Jaundice*—Five of the twelve patients in our series had a positive thymol turbidity test. Four of the five patients with positive tests were shown to have cholangitis in the presence of obstruction, and the same condition was suspected in the fifth positive case

*Diseases of the Gall Bladder*—This group of eleven patients included cases of acute and chronic cholecystitis as well as cholelithiasis. In six of them the thymol turbidity test was positive. It seems possible that the positive results were due to an associated cholangitis

*Diseases With Widespread Liver Destruction*—Eleven such cases were tested in the series, these included primary neoplastic disease of the liver, massive metastatic infiltration of the liver, and one case of massive infarction of the liver. Eight of the eleven patients had a positive thymol turbidity test

*Therapeutic Malaria*—Nine patients undergoing malarial fever therapy for neurosyphilis were tested. All of them had strongly positive thymol turbidity tests, with increasing values according to the duration of the fever therapy. This is in agreement with the results of other observers who have noted that other tests of liver function give evidence of liver damage.<sup>9, 11</sup>

*Acute Lymphogranuloma Venereum*—This group of twenty-one cases was unusual in that the virus of lymphogranuloma venereum had been isolated in every instance.<sup>12</sup> All but one of the patients had a positive thymol turbidity test. MacLagan in his original work included one case of lymphogranuloma venereum and that patient had a strongly positive thymol turbidity test

*Rheumatoid Arthritis*—Seventeen patients with acute and chronic rheumatoid arthritis were tested. Fourteen of them had a positive thymol turbidity test. The average number of units was 10. Carter and MacLagan<sup>13</sup> obtained positive results in thirteen of thirty-four patients with rheumatoid arthritis

*Acute Rheumatic Fever*—Six of eleven patients with acute rheumatic fever had weakly positive thymol turbidity reactions. The average number of units was 6.7

*Heart Disease With and Without Failure*—Approximately half of the patients with various forms of organic heart disease had positive thymol turbidity tests. The presence or absence of circulatory failure did not appear to influence the result. Carter and MacLagan found that ten of twenty-eight cases or 36 per cent of their patients with congestive heart failure, had positive thymol turbidity tests

*Chronic Lung Disease*—There were twenty-eight patients in this group which included such entities as bronchiectasis, empyema, pulmonary fibrosis, asthma, chronic emphysema, lung abscess, and chronic pulmonary tuberculosis. Fifteen of them had a positive thymol turbidity reaction. Five of the six patients with bronchiectasis gave positive tests

*Acute Infectious Diseases*—Seven of sixty-seven patients with acute infectious diseases were tested and 37.3 per cent showed a positive reaction. The

incidence seemed notably high in miliary tuberculosis, secondary syphilis, infectious mononucleosis, tularemia, and bacterial endocarditis. The highest value in the entire series, namely 53 thymol turbidity units, was found in the serum of a patient with miliary tuberculosis. Since infectious mononucleosis may be accompanied by a hepatitis it is not surprising to find the thymol turbidity test positive during the acute phase of this disease. MacLagan<sup>2</sup> found that six of seven patients with subacute bacterial endocarditis had a positive thymol turbidity test. Negative results were obtained in such diseases as lobar pneumonia, typhus, pharyngitis, tuberculous peritonitis, and so on.

*Neoplastic Diseases*—Fifteen patients were studied and seven had a positive thymol turbidity test. This group included three cases of bronchiogenic carcinoma and three of carcinoma of the colon.

*Controls*—Seventy-four members of the medical and nursing staffs were included in this group. Six, or 8 per cent, had positive thymol turbidity tests, ranging between 5.0 and 9.5 units. Most other workers<sup>1, 8, 14</sup> have reported that the normal control subjects did not give values above 4.0 to 4.7 thymol turbidity units. Ley and co-workers<sup>15</sup>, however, determined their normal values for the thymol turbidity test statistically and concluded that the upper limit of normal was 8.7 units. Using this figure as the maximal normal level, 19 per cent of their controls had an elevated thymol turbidity test. It is of interest to note that the thymol turbidity test in 8.5 per cent of their controls exceeded 9 units.

#### COMPARISON OF THYMOL TURBIDITY TEST WITH CEPHALIN FLOCCULATION TEST

Although there is both experimental and clinical evidence that the thymol turbidity and cephalin flocculation tests depend on different factors, it was of interest to compare the two tests in our series since both are generally regarded as liver function tests. Table II shows the comparative results of the two tests in some of the larger groups of patients in the present series of cases.

TABLE II COMPARISON OF THE THYMOL TURBIDITY AND CEPHALIN FLOCCULATION TESTS

GROUP	NUMBER OF CASES	TT + CF +	TT + CF -	TT - CF +	TT - CF -
Infectious hepatitis	36	27	7	1	1
Cirrhosis	48	37	9	1	1
Obstructive jaundice	12	2	3	1	0
Lymphogranuloma venereum	21	19	1	1	-
Neurosyphilis with fever therapy	9	9	-	-	-

*Infectious Hepatitis*—Results of twenty-eight of the thirty-six cases agreed in both tests. There were seven tests in which the thymol turbidity was positive and the cephalin flocculation negative and one in which the cephalin flocculation was positive and the thymol turbidity negative. This is in agreement with other investigations, indicating that the thymol turbidity test is more sensitive than the cephalin flocculation test in hepatitis.<sup>2, 16, 17</sup> Repeated examinations of our patients with the two tests also indicated that the thymol turbidity was a better test for following the progress of infectious hepatitis than the cephalin flocculation, since it remained positive as long as there were any symptoms of the disease. An example in a case of infectious hepatitis is illustrated in Fig. 1.

The patient was a 46 year old white woman who entered the hospital in the acute phase of infectious hepatitis. At the time of entry the thymol turbidity was 43 units and the cephalin flocculation 4 plus. The thymol turbidity fell rapidly. The patient was allowed to be out of bed thirty five days after admission before the thymol turbidity had reached a "normal" level. Within a few hours she complained of nausea and pain in the right upper quadrant. A thymol turbidity taken at this time showed a rise from 8 to 26 units. Bed rest was resumed and one day later the thymol turbidity fell to 11 units while the cephalin flocculation was 2 plus. Ninety days after admission the thymol turbidity was normal. This was forty five days after the cephalin flocculation had become negative.

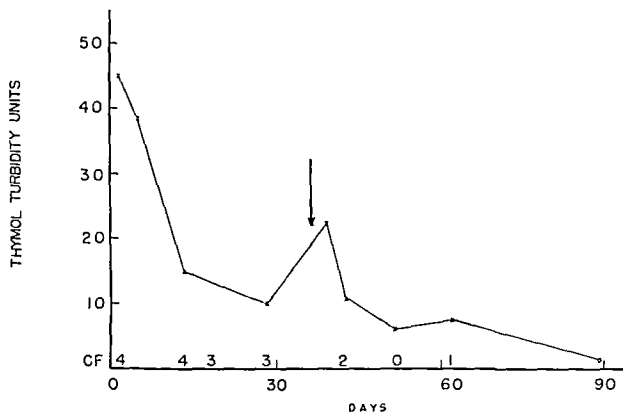


Fig 1—Infectious hepatitis

This case emphasizes the usefulness of the thymol turbidity test in determining the length of convalescence in infectious hepatitis. Correlation with liver biopsy seems to indicate that the thymol turbidity test is the most accurate index of recovery from this disease.

**Cirrhosis**—The two tests agree in a majority of the cases. However, here also the thymol turbidity test appeared to be more sensitive than the cephalin flocculation test. There were nine cases in which the thymol turbidity was positive while the cephalin flocculation was negative. In contrast, there was only one case with a positive cephalin flocculation and a negative thymol turbidity test.

**Obstructive Jaundice**—In this series of only twelve cases it is not possible to draw conclusions about the relative usefulness of thymol turbidity and cephalin flocculation tests. Neither test can be relied upon to rule out this diagnosis. Their greatest usefulness seems to be in cases of jaundice with a negative thymol turbidity or cephalin flocculation. In such circumstances the chances are against the likelihood of hepatitis. However, a positive thymol turbidity or cephalin flocculation test does not rule out obstructive jaundice. It appeared that the cephalin flocculation was a slightly better diagnostic aid than the thymol turbidity test in this syndrome since the thymol turbidity test was positive in cases with cholangitis in which the cephalin flocculation test was negative.

*Lymphogranuloma Venereum*—It should be pointed out that the high incidence of positive results with both tests shown in this report applies to acute cases. It is probable that in chronic lymphogranuloma venereum the incidence of positive tests would not be as great. Nevertheless the possible existence of this disease must be considered when the test is employed in colored patients.

*Therapeutic Malaria*—Both tests were positive in all nine cases. The results here are essentially similar to those which have appeared in the literature.

TABLE III COMPARISON OF THYMOL TURBIDITY AND CEPHALIN FLOCCULATION TESTS

	NUMBER OF CASES	TT + CF +	TT + CF -	TT - CF +	TT CF
Total	567	29%	25%	6%	40%

Table III presents a comparison of the thymol turbidity and cephalin flocculation tests of our entire series. Including the control cases there were 361 cases studied. The thymol turbidity and cephalin flocculation tests agreed in 69 per cent of the cases. The thymol turbidity was positive and the cephalin flocculation negative in 25 per cent of the cases, the thymol turbidity was negative and the cephalin flocculation positive in 6 per cent of the cases. Therefore, it would seem that the thymol turbidity test is considerably more sensitive than the cephalin flocculation test.

TABLE IV COMPARISON OF THYMOL TURBIDITY AND CEPHALIN FLOCCULATION TESTS IN RARE DISEASES

DIAGNOSIS	TT	CF
Disseminated lupus erythematosus	23	-
Disseminated lupus erythematosus	16	-
Dermatomyositis	23 5	3+
General myositis	17 5	3+
Scleroderma	21 5	3+
Calcinosis universalis	12 5	-
Pemphigus	10	-
Pemphigus	-	-
Porphyria	-	-
Hypoprothrombinemia	-	-
Elephantiasis	10 5	-

Table IV shows the results of the thymol turbidity and cephalin flocculation tests in some rare diseases. There were two cases of disseminated lupus, both strongly positive with the thymol turbidity and negative with the cephalin flocculation. There was one case each of dermatomyositis, generalized myositis (possibly dermatomyositis, but with a negative skin and muscle biopsy), scleroderma and calcinosis universalis (due to dermatomyositis). Both tests were positive in three of the four cases and in the fourth case (calcinosis universalis) the cephalin flocculation was negative. One case of pemphigus gave a negative result with both tests, whereas in a second case the thymol turbidity was positive and the cephalin flocculation negative. One case of porphyria and one of idiopathic hypoprothrombinemia had negative results with both tests.



## COMMENT

It appears from the results just presented that the thymol turbidity test cannot be regarded solely as a test of liver function. Positive results were frequently obtained in diseases in which there was no other evidence of liver dysfunction. Such diseases included rheumatic fever, congestive heart disease, lymphogranuloma venereum, carcinoma without liver involvement, and so on. The thymol turbidity test should be looked upon only as a measure of abnormal serum protein pattern, not necessarily related to liver function. It is conceivable that many conditions other than liver disease might occasion a change in the pattern of the serum proteins. Evidence in favor of this hypothesis is offered by the number of positive thymol turbidity tests in the control series of apparently normal healthy young men and women of the professional staffs.

In cases of liver dysfunction, the thymol turbidity test is a rather sensitive test in such diseases as infectious hepatitis, cirrhosis, Weil's disease, malaria, and so forth. While it is a great aid in diagnosis of these conditions, it can be used to follow the progress only of infectious hepatitis and probably Weil's disease. Labby and co-workers<sup>18</sup> have shown that neither the thymol turbidity nor the cephalin flocculation test is of value in following the progress of cases of cirrhosis. This is unfortunate since the thymol turbidity test is a quantitative test and can be performed easily.

There are certain features of rheumatoid arthritis that is palmar erythema, the remission of symptoms with the onset of jaundice, and the positive liver function studies,<sup>19</sup> which seem to indicate dysfunction in the liver in rheumatoid arthritis. The high percentage of positive thymol turbidity tests in this disease adds another link in the circumstantial evidence of such a relationship.

It has long been recognized that lymphogranuloma venereum is a generalized disease. The incidence of this disease is rather high among Negroes. Beeson and Miller<sup>20</sup> have shown that many Negroes have abnormal serum protein reactions as evidenced by positive formol gel reactions. They postulated the possibility that these two findings were related. The high incidence of positive thymol turbidity and cephalin flocculation tests in lymphogranuloma venereum tends to confirm this hypothesis.

## SUMMARY

The sera of 567 individuals were studied in order to evaluate the thymol turbidity test. The results indicate that the thymol turbidity test should not be regarded as a specific test of liver function. It appears to depend upon abnormal protein patterns which may or may not reflect liver disease. Many conditions in which all other tests of liver function are normal may have a positive thymol turbidity test.

The test is of greatest value in following the progress of cases of infectious hepatitis.

Both thymol turbidity and cephalin flocculation are frequently positive in lymphogranuloma venereum and rheumatoid arthritis, thus necessitating cautious evaluation of the tests in the presence of these diseases.

Negative thymol turbidity and cephalin flocculation tests are helpful in the differential diagnosis between obstructive jaundice and hepatogenous jaundice. Positive results, however, should not be relied upon to rule out the possibility of obstructive jaundice. The thymol turbidity is frequently positive in obstruction is complicated by cholangitis.

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## ETHYLENE GLYCOL POISONING

### A CLINICAL AND PATHOLOGIC STUDY OF THREE CASES

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CONFUSION in the layman's mind over the varying composition of anti freeze solutions has led to the accidental poisoning of an increasing number of individuals. Under these circumstances ethylene glycol solutions (for example, Prestone) have been ingested with serious results in each instance. Such accidents might be avoided to some extent if containers of ethylene glycol solutions were more conspicuously labeled Poison.

Ethylene glycol ( $\text{HOCH}_2\text{CH}_2\text{OH}$ ) is a colorless odorless liquid which has a characteristic pleasant bittersweet flavor. In vivo ethylene glycol is oxidized to oxalic acid and then to glycollic acid. Its toxicity has been studied rather extensively experimentally by a number of workers.

*Experimental Data*—Page<sup>1</sup> studied the effects of ethylene glycol in dogs, rabbits, and rats, and in fatal poisoning he found hemolysis of blood, bloody urine, and distended urinary bladders. Comparing the toxicities of ethylene glycol, propylene glycol, and diethylene glycol, Holec<sup>2</sup> found that animals died twice as fast with ethylene glycol. Laug and co-workers<sup>3</sup> describe weakness, lack of coordination, coma, and death in animals fed ethylene glycol. Pathologic studies revealed hydropic degeneration of convoluted tubules and focal necrosis of the liver. In addition, pulmonary congestion and hemorrhage were noted as well as hemorrhages in the stomach. They found the  $\text{LD}_{50}$  of ethylene glycol to be about half that of diethylene glycol in most animals. These results were confirmed by Smyth, Seaton, and Fischer.<sup>4</sup>

Kesten and co-workers<sup>5</sup> described experimental renal lesions with calcium oxalate deposits and high blood nonprotein nitrogen values following ethylene glycol administration. Similar lesions were observed by Morris and associates<sup>6</sup> in rats, including the formation of calcium oxalate bladder stones.

Wiley and co-workers<sup>7</sup> recognized the conversion of ethylene glycol to oxalic acid in experimental animals but did not feel that such conversion occurred in amounts sufficient to explain its toxicity. Similar data are presented by Muhnos and co-workers<sup>8</sup> who found crystalline deposits only in chronic toxicity experiments. Administration of sodium oxalate in appropriate amounts did not cause death in experimental animals. They found no hydropic degeneration of kidney tubules and felt that animals died of extrarenal causes. Wiley<sup>7</sup> described lymphocyte meningeal reactions in experimental animals following ethylene glycol administration. Newman and associates<sup>9</sup> perfused livers with ethylene glycol and found oxygen consumption depressed and lactic acid formation increased.

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Experimental evidence thus suggests that ethylene glycol acts as a depressant to the central nervous system and produces lesions in the kidney, liver, and lung

*Clinical Data*—Bachem (1917)<sup>10</sup> drank ethylene glycol and observed an increase in excretion of oxalic acid in the urine. In 1927 Page<sup>1</sup> drank 15 cc diluted without effect

In 1930 Hausen<sup>11</sup> reported two cases with recovery. Decapsulation of the kidney was performed in one instance. In the same year Biecke<sup>12</sup> reported two cases successfully treated with unilateral renal decapsulation. Three additional cases have been reported in brief<sup>13, 14</sup>

In 1943 Boemke<sup>15</sup> reported four fatal cases with necropsy findings. Two of his patients exhibited hyperemia of the brain and leptomeninges, small ring hemorrhages in the brain stem, and perivascular cell infiltrates in the gray and white matter of the cerebrum. Sections of the kidneys revealed dilated tubules with fat droplets in the epithelium. The lumen of tubules contained hyaline material, crystals, and red blood cells. Boemke attributed changes in the brain to previous typhus infections.

A clinical and pathologic report of eighteen fatal cases was published by Pons and Custer<sup>16</sup>. The clinical description of these cases emphasized the predominance of the neurological manifestations, that is, coma, convulsions, and so on. The urine contained oxalate crystals in each recorded instance. All tissues were congested. Calcium oxalate crystals were seen in all kidney sections and in occasional brain sections. The authors emphasized the inflammatory reaction seen perivascularly in the brain and diffusely in the meninges. In some instances hemorrhage accompanied this infiltration. Degenerative changes in cells of the brain were also found. These authors felt the lesions in the central nervous system explained the fatal outcome in acute severe ethylene glycol poisoning.

Another case with clinical and pathologic findings has been reported in detail by Milles<sup>17</sup>. This patient was euphoric and later depressed, he developed hypertension and fever and died twenty-two hours following the ingestion of ethylene glycol. Autopsy revealed pulmonary edema, injection of pial vessels, and swollen and vacuolated liver cells. The glomerular capillaries were distended and tubular epithelium was swollen. Crystals were seen in the tubules. The acute course was attributed to oxalate poisoning, and several therapeutic suggestions were given with this in mind.

#### CASE PRESENTATIONS

The three patients herein presented were admitted to the station hospital at Los Alamos, N. M., following a drinking bout during which one of the victims induced his friends to share his "wine". The quantities consumed could not be estimated with any accuracy. The material was consumed from three to five hours prior to admission.

CASE 1—A. B., a 36 year old Spanish American, was admitted to the hospital in coma about three hours after he had ingested the "green wine."

*Physical Examination* The temperature was 101 F, pulse rate 128, and respiratory rate, 44. Slight cyanosis was present. Greenish froth exuded from the nose and mouth. The pupils reacted to light. Heart, lungs and abdomen were negative. Blood pressure was 100/90. Reflexes were absent and extremities were flaccid.

*Laboratory Examination* The red blood count was 5,350,000 hemoglobin content, 14.5 Gm. per 100 c.c., and white blood count 32,300. Differential white cell count was as follows: neutrophils, 91 per cent, stab forms, 4 per cent, metamyelocytes, 1 per cent, lymphocytes, 1 per cent, monocytes, 3 per cent. Urinalysis showed a specific gravity of 1.009, acid reaction, a trace of albumin, 2 plus sugar (glucose iv), numerous red blood cells and a few crystals resembling hippuric acid. Spinal fluid was slightly cloudy and contained 214 white blood cells per cubic millimeter, globulin was 4 plus and total protein was 170 mg per cent. Blood nonprotein nitrogen was 81 mg per cent and carbon dioxide combining power was 7.5 volumes per cent.

*Course* The stomach was immediately lavaged with 4 per cent solution of sodium bicarbonate, and 500 c.c. of this solution were left in the stomach. One liter of 10 per cent glucose in distilled water and 1 liter of one sixth molar sodium lactate in normal saline were given intravenously. Oxygen was given by nasal catheter. The patient remained unconscious, the pulse became very rapid and weak and the respirations rapid, deep and labored. The nasal tube was inserted twelve hours after admission and 2 Gm. of sodium bicarbonate were given by tube each hour for six doses. A series of generalized convulsions began at this time and the blood pressure fell to 60/20. Magnesium sulfate was given intramuscularly in an effort to control convulsions. An additional 2 liters of one-sixth molar sodium lactate were given. The patient expired twenty hours after admission.

CASE 2—M S, a 27 year old Spanish American, was found unconscious in a furnace room, where he evidently had collapsed while attempting to carry out his duties as a fireman. Approximately three hours previously he was reported to have consumed some green liquid with the first patient.

*Physical Examination* Temperature was 98 F, pulse rate, 90, respiratory rate, 32, and blood pressure 140/90. The patient was comatose but reacted slightly to painful stimuli. Pupils were dilated but reacted to light. Heart, lungs and abdomen were not notable. The limbs were flaccid and reflexes were absent.

*Laboratory Examination* Blood count revealed 5,820,000 red blood cells, 18.8 Gm hemoglobin, and 37,200 white blood cells. The differential white cell count was as follows: neutrophils 85 per cent, stab forms, 4 per cent, lymphocytes 1 per cent, monocytes 10 per cent. Urinalysis showed a specific gravity of 1.009, acid reaction, a trace of albumin and sugar, 3 to 5 red blood cells per high power field, and numerous crystals resembling hippuric acid. The spinal fluid was slightly cloudy and contained 62 white blood cells per cubic millimeter. Globulin was 4 plus and total protein, 170 mg per cent. Blood nonprotein nitrogen was 60 mg per cent, and carbon dioxide combining power, 7.5 volumes per cent.

*Course* Therapeutic efforts were identical with those described in Case 1, namely gastric lavage, liberal administration of fluids, alkali, and oxygen. The patient remained comatose, the pulse rate rose to 136 and the respiratory rate to 36. Breathing was deep, labored and rapid. Blood pressure rose to 190/105. Generalized convulsions began fifteen hours after admission, following which the patient failed rapidly with a fall in blood pressure to 70/40. The patient expired seventeen hours after admission.

CASE 3—P B, a 53 year old Spanish American, was brought to the hospital for observation because he had consumed one drink of the same liquid. The patient was at work when contacted and except for appearing slightly intoxicated and unsteady was in satisfactory condition. In addition to the drink, which was consumed eleven hours before admission, the patient had had several bottles of beer and several "shots" of whiskey during the day. There had been no nausea, vomiting or other untoward symptoms.

*Physical Examination* The rectal temperature was 98° F, pulse rate, 130, and respiratory rate, 24. The patient was well oriented, cooperative, and did not appear ill. Conjunctival vessels were injected. The pupils were small and reaction to light was questionable. No other abnormalities were noted.

*Laboratory Examination* Red blood cell count was 4,980,000, hemoglobin content 12.4 Gm per 100 cc, white blood cell count, 40,000. Differential white cell count revealed neutrophils, 89 per cent, stab forms, 10 per cent, lymphocytes, 3 per cent, and monocytes, 5 per cent. Urinalysis showed a specific gravity of 1.009, acid reaction, no albumin or sugar, there were numerous red blood cells and crystals resembling hippuric acid. Stomach content showed no free hydrochloric acid and 4 plus occult blood. Spinal fluid contained 39 white blood cells per cubic millimeter, 4 plus globulin, and total proteins of 124 mg per cent. Blood nonprotein nitrogen was 59 mg per cent and carbon dioxide combining power, 3 volumes per cent.

*Course* Three hours after admission the patient became restless and lapsed into coma. Oxygen was administered and 1 liter of one sixth molar sodium lactate was given intravenously. Respirations were shallow and rapid. Blood pressure was 195/80. Nine hours after admission respirations became very labored and shortly thereafter the patient became cyanotic and respirations ceased. The patient was revived with artificial respiration, adrenalin, and Coramine, but remained comatose. The pulse remained rapid and weak and respirations were deep and labored. A second liter of one sixth molar sodium lactate was given. Sodium bicarbonate (2 Gm) was given by nasal tube every two hours for four doses. The patient expired seventeen hours after admission.

### *Autopsy Findings—*

*Gross Pathology* The external appearance in all three instances was not remarkable. Livor mortis and a moderate degree of rigor mortis were present. Following the primary incision, in each case the urinary bladder was found to be greatly distended to the level of the umbilicus and filled with clear, pale urine. In all three, the gross appearance of the organs was nearly identical save for the presence of active tuberculous pulmonary lesions in Cases 1 and 2.

All organs showed moderate to severe engorgement of vessels. Although the peritoneal surfaces were not remarkable, the pleural surfaces, both visceral and parietal, were studded with petechial hemorrhages, hemorrhagic streaks, and several large subpleural hematomas. The lungs were markedly congested, more so in the posterior dependent portions, and on cross section revealed multiple small hemorrhagic areas scattered throughout the parenchyma, varying in number in each case. The pericardium was of normal appearance, but the epicardial surface was streaked with large, recent hemorrhages sometimes reaching the proportions of hematomas. These hemorrhages were found principally along the coronary vessels and their branches and over the auricles. In one case tiny hemorrhages were observed in the adventitia of the superior vena cava, near the heart, in another, similar petechial hemorrhages were seen in the adventitia of the ascending aorta. The relative size of the hearts was not remarkable and no cardiac dilatation was noted. Grossly the myocardium was not significantly altered, but in one case small hemorrhages were seen in the endocardium.

The stomachs and intestines were not remarkable, except for congestion and occasional small mucosal hemorrhages. The mucosa of the esophagus, in each case, was smoky-gray in color with one or two small points of epithelial sloughing. The livers were not enlarged, they were deep reddish brown in color with

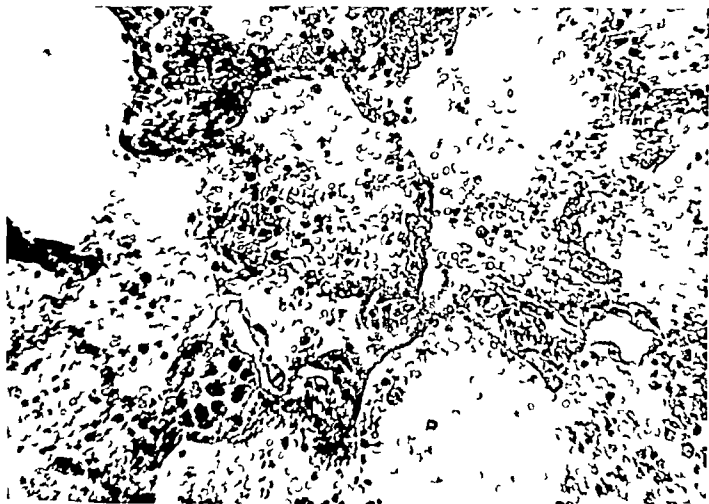


Fig 1—Section of lung through hemorrhagic area showing deposition of fibrin like material lining the alveolar walls and extensive extravasation of red cells into the alveolar spaces (X100)



Fig 2—Section of superficial myocardium showing extensive recent extravasation of blood (X100)

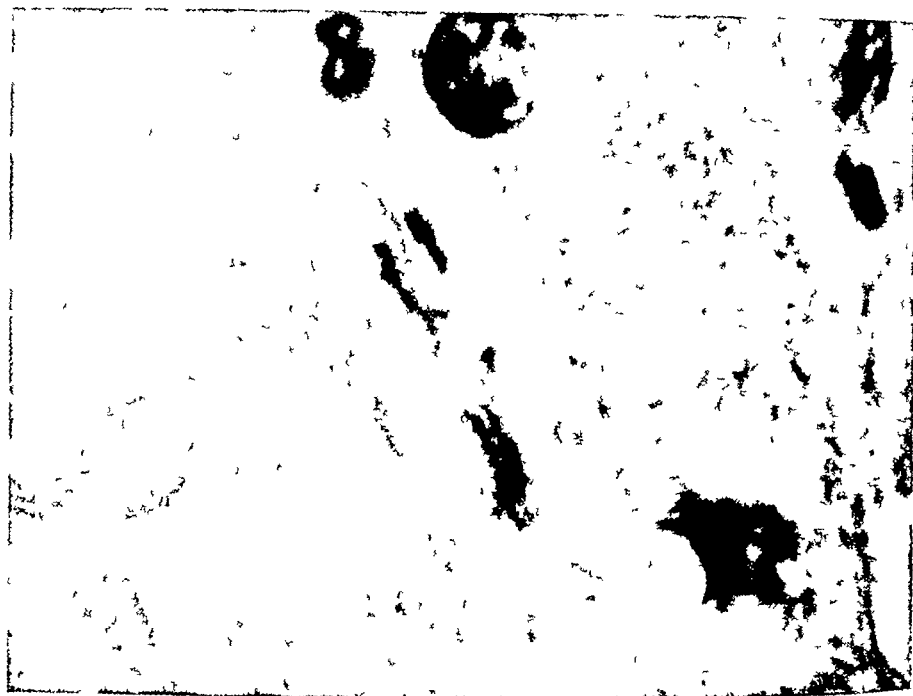


Fig 3—Liver showing marked perisinusoidal edema containing albuminous deposits ( $\times 90$ )

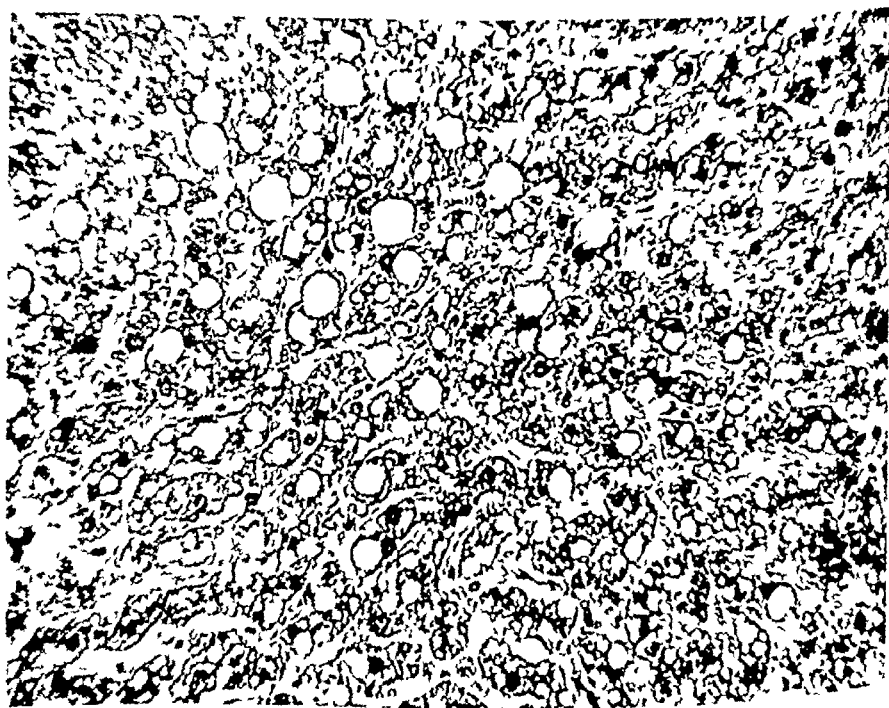


Fig 4—Liver showing marked fatty infiltration ( $\times 100$ )



distinct markings, but showed no other significant changes. The kidneys were of normal size and markedly congested, and in one a few petechial hemorrhages were noted under the capsule. Small hemorrhages were seen in the pelvic lining of all and in the bladder mucosa of two.

The brain, in each case, showed marked vascular engorgement with scattered petechial hemorrhages over the surfaces and within the sulci. The cerebrospinal fluid was grossly clear and the ventricles were not dilated. Coronal sections at 10 cm intervals showed only congested and dilated vessels in two instances, and in a third, a few scattered petechial hemorrhages in the white matter. In two instances, recent hemorrhage was present in the mastoid cells.

*Histopathology* The microscopic pictures of all three were relatively similar to one another except for variations in degree which will be mentioned. No space will be given to description of the tuberculous lesions of the lungs. Tissue sections from the lungs revealed moderate alveolar distension, emphysema and variable degrees of capillary congestion especially in the dependent portions of the lungs. In all lobes many small and large areas of recent hemorrhage within the parenchyma and subpleurally were noted. The alveolar walls intermittently presented a uniform pink staining material having a fibrinoid appearance (Fig 1). This material formed heavy deposits lining the alveolar walls, sometimes sloughing off into the alveolar spaces. Some of the capillary endothelial cells were swollen and in many places actually appeared degenerate and became contiguous with the fibrin like deposits. In Cases 2 and 3 congestion and hemorrhage were marked but the fibrin like deposits were seen only in scattered places.

Sections of the myocardium in each case showed many large recent extravasations of red cells under the epicardial membrane and scattered throughout the myocardium between the muscle fiber bundles (Fig 2). The vessels again were markedly dilated and very occasionally hyaline like degeneration of the capillary wall similar in character to that of the pulmonary capillaries was found. There were areas in which degenerative changes of the myocardial fibers had taken place consisting of interstitial edema, swelling of the fibers and loss of cross striations. A fat stain (sudan II) revealed a diffuse deposit of finely granular lipid substance in the muscle fibers.

Although grossly the livers showed no specific changes the sections evinced a fairly well preserved architecture with the presence of wide zones of edema between the liver cord cells and the venous sinusoids. In most instances, these wide edematous spaces contained variable quantities of amorphous albuminous material (Fig 3). The liver cells although well preserved were filled with many large vacuoles. Sudan II staining revealed numerous fat droplets in the cytoplasm of the liver cells. This was especially striking centrally, but affected about three fourths of the lobule (Fig 4). Sections of the spleen showed foci of hemorrhage and diffuse congestion.

Sections of the esophagus revealed rather well preserved epithelium which was raised from its base and interrupted in two or three places. An occasional nucleus appeared pyknotic but for the most part the cells were normal and stained well. The immediate subepithelial layers were intensely congested with

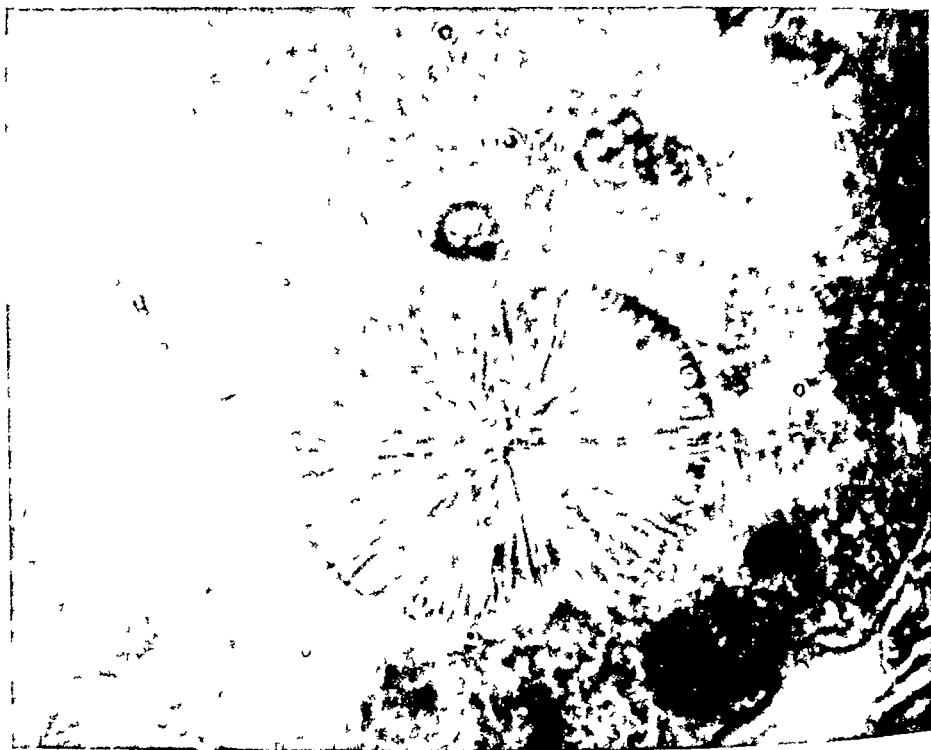


Fig 5—Tufted crystalline deposits in renal tubule. Note the well preserved epithelial lining ( $\times 970$ )

a few small hemorrhages. The vessels, however, did not show any evidence of degeneration or focal necrosis. Sections of the stomach and small and large intestine were not especially remarkable. There was marked capillary dilatation, engorgement, and edema of the lamina propria. Occasionally small hemorrhages were seen.

The kidneys showed the most striking distention of capillaries, arterioles, and venules alike. In many instances, there were small amounts of blood extravasated outside the vessel walls. The glomeruli were large but without proliferation of epithelium or endothelium; here, too, there was marked distention of the capillary loops forming lakes of blood which could be interpreted as true stasis. There was no evidence of capillary degeneration nor of hyaline necrotic mural changes such as were seen in the lung. Although the lining epithelium was well preserved, approximately two thirds of the renal tubules contained large grayish crystals, many appearing as shocks of wheat with a central binding similar to sulfathiazole crystals but smaller (Fig 5), others showed irregular plate-like appearance similar to uric acid crystals. Positive identification of these crystals was not made.

Numerous sections were taken from various portions of the brain (the frontal and occipital lobes, the internal capsule, cerebellum and dentate nucleus, pons, medulla, and olive) and spinal cord. The changes in nearly all

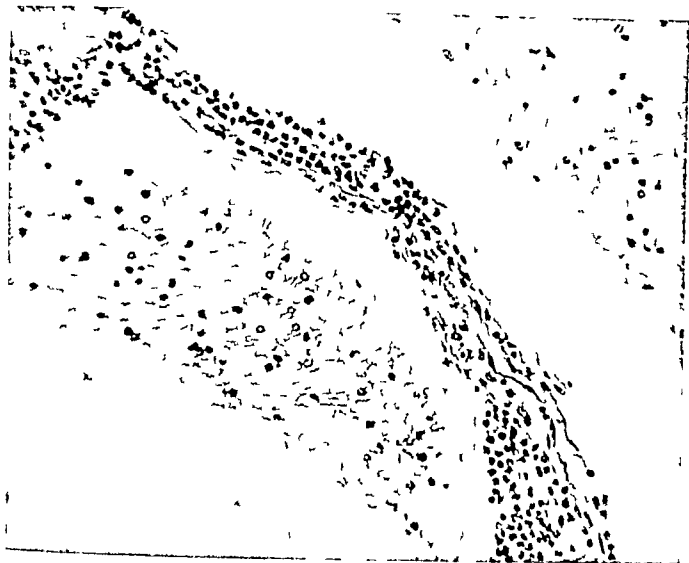


Fig 6—Meninges. The thickening of the pia arachnoid is chiefly perivascular consisting of predominantly neutrophilic leucocytes moderate numbers of lymphocytes and pale monocytes and extravasated red cells (X100)

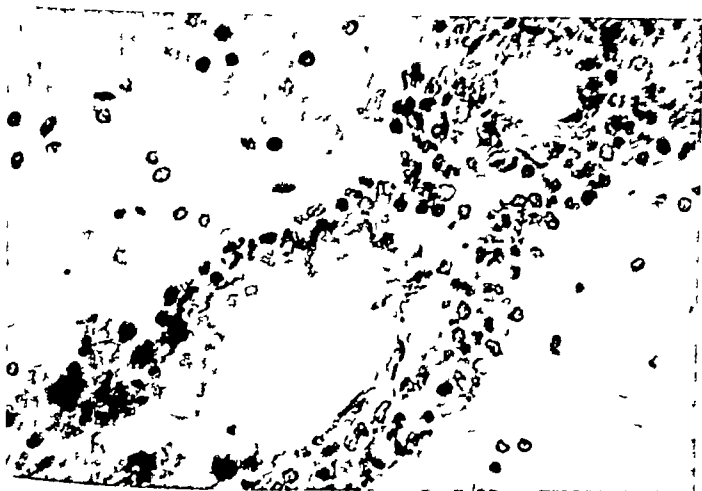


Fig —Small cerebral vessels showing perivascular edema and intensive diapedesis of leucocytes and red cells (X430)

sections included simple vascular congestion, the diapedesis of leucocytes into the perivascular spaces, the extravasation of red cells, and frank exudative meningo-encephalitis (Fig 6). Some vessels showed only moderate numbers of leucocytes infiltrating through the vessel walls and filling the perivascular space. This infiltrate consisted chiefly of neutrophils with varying numbers of lymphocytes and palely stained mononuclear cells. Other vessels showed perivascular infiltrations of equal numbers of leucocytes and red cells (Fig 7). Occasionally, however, only red cells were seen about the vessels, sometimes in ring form. In only one of these cases (Case 1) was the pia-arachnoid found to be universally infiltrated with extravasated blood, serum, and leucocytes, principally neutrophils. In this case, the superficial vessels of the parenchyma also showed a marked perivascular cuffing with neutrophils, lymphocytes, and occasional monocytes. In the remaining two cases only few focal areas of meningeal infiltration were seen, the principal changes being congestion, edema, and focal hemorrhages. These vascular changes were found in all sections of the brain stem and in the spinal cord as well. Some of the small vessels were filled with hemolyzed blood cells. Curiously enough, in one section of the internal capsule (Case 2) several of the small capillaries exhibited crystalline tufts exactly similar to those found in the renal tubules. With toluidine blue stain, in most instances, the ganglion cells appeared to be well preserved and arranged perpendicularly to the brain surface. However, in each case there were scattered focal areas of derangement of these large cells which stained deeply and appeared somewhat pyknotic. Occasionally chromatolysis as well as satellitosis with oligodendroglia was observed. Early chromatolytic changes of the ganglion cells of the spinal cord were also seen. Myelin stains showed no abnormality, and modified Spielmeyer's stain of peripheral nerves showed no evidence of demyelination of nerve fibers.

Sections of thoracic and abdominal lymph nodes, aorta, bone marrow, prostate, testis, adrenals, thyroid, pituitary, and peripheral nerves showed no significant alterations.

TABLE I ETHYLENE GLYCOL CONTENT OF TISSUES AND URINE  
(EXPRESSED IN MILLIGRAMS PER CENT)

	CASE 1	CASE 2	CASE 3
Brain	500	450	400
Liver	133	100	662
Kidney	410	440	500
Urine	1650	1000	1000

*Chemical Laboratory Analysis* Portions of brain, liver, and kidney were analyzed for presence or absence of common poisons and ethylene glycol. Equal samples in each case were submitted as well as samples of the urine and gastric contents. The post-mortem gastric contents were negative. The tissue sections were entirely negative for the common volatile poisons, alkaloids, and heavy metals. Special procedures were instituted for the qualitative and quantitative assay of ethylene glycol and the results are incorporated in Table I.

Technique employed for detection of ethylene glycol in body tissues and urine

**Brain, Liver, and Kidney** Twenty five grams of tissue were chopped finely with scissors and transferred to a 500 ml side arm distilling flask. 20 to 25 cc distilled water were added. Steam distillation was carried out until 30 cc of distillate were obtained. The residue was centrifuged and the total volume measured and made up to 50 cubic centimeters. Two cubic centimeters of 2/3 N sulfuric acid and 2 cc 10 per cent sodium tungstate were added to 16 cc of the supernatant fluid. To 5 cc of the filtrate were added 2.5 cc 2 per cent potassium permanganate and 0.6 cc concentrated sulfuric acid. After standing five minutes, 0.5 cc 10 per cent oxalic acid and 0.6 cc concentrated sulfuric acid were added. After the solutions became colorless 5 cc Schiff's reagent were added. After fifteen minutes the unknowns were compared with standards that were prepared simultaneously. Normal body tissues were employed as controls. These were uniformly negative for ethylene glycol.

**Urine** Urine was tested in a similar manner. One cubic centimeter urine plus 4 cc distilled water were used instead of the 5 cc filtrate employed in tissue analysis. Normal controls were again negative.

Formaldehyde was detected in urine by adding 1 cc Schiff's reagent to 3 cc distilled water and 1 cc urine. The pink colors were compared with standards prepared simultaneously.

The urine also was found to contain small quantities of formaldehyde (presumably an oxidation by product) in the following amounts:

Case 1	40 mg per cent
Case 2	52 mg per cent
Case 3	44 mg per cent

The heaviest concentrations of the specific agent in the brain and in the urine were found in Case 1. Correspondingly, the tissue sections showed the most dominant lesions histologically.

#### DISCUSSION

The bottle from which these patients drank an unknown quantity was shown by analysis to contain a 40 per cent solution of ethylene glycol. Tests for methyl alcohol on the same liquid were negative.

The picture presented by all three patients was strikingly similar being characterized by coma, acidosis, hypertension, convulsions, and death. The widespread toxic reaction was evidenced clinically by the extreme leucocytosis, albuminuria, microscopic hematuria, nitrogen retention, lowered carbon dioxide combining power and spinal fluid changes. The therapeutic efforts had no apparent beneficial effect.

The pathogenesis of ethylene glycol toxicity in man is not clearly understood. Pons and Custer<sup>10</sup> attributed the main effect to a chemical meningitis and Milles<sup>17</sup> to ovalate poisoning. The evidence presented in these three cases seems to indicate widespread capillary damage. This is assumed to be the primary lesion. Widespread hemorrhages result as seen in pleura, lung, heart, pericardium, kidney, and brain. In all three, the brains and vessels were congested with perivascular infiltration of leucocytes and red blood cells. In Case 1 the changes in the meninges and brain were more diffuse and well developed, suggesting a chemical meningoencephalitis. The edema and fat infiltration seen in the liver were striking and presumably represent a direct toxic effect. Crystals (presumably calcium oxalate) seen in kidney tubules and in the brain were considered a characteristic but unimportant finding.

The demonstration of ethylene glycol in body tissues and in post mortem urine serves to demonstrate its wide distribution corresponding with the diffuse pathologic lesions. The high concentration of the chemical in the urine re-emphasizes that the principal path of excretion is via the kidney.

Specific therapeutic measures do not seem to be at hand.

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# THE ACTION OF PILOCARPINE ON THE LUNGS IN NORMAL AND ASTHMATIC SUBJECTS

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THE reaction of the tracheobronchial tree to histamine and acetyl-beta-methylcholine (Mechoyl chloride) in normal and asthmatic subjects has been described by one of us (J. J. C.) in previous reports.<sup>1,2</sup> While it was shown that in the latter subjects the parenteral injection of either histamine or Mechoyl chloride induced asthma-like attacks, with reduction in the vital capacity, it appeared that the respiratory tract was more reactive to Mechoyl chloride than to histamine in many of the cases. In view of the implication of these findings with regard to the etiology and possibly the therapy of spontaneous asthma, it was felt that further study of the role of the parasympathetic nervous system in bronchial asthma was warranted. The present communication deals with the action of a parasympathomimetic drug, pilocarpine, on the tracheobronchial tree in normal and asthmatic subjects. Comparison of the respiratory reaction after pilocarpine with that following Mechoyl chloride, and in some instances Prostigmine, is also made in an attempt to furnish further information on the pharmacodynamic action of pilocarpine.

Few studies on pilocarpine have been made in man except those dealing with the action of the drug on the sweat glands. This substance acts on cells innervated by postganglionic cholinergic fibers and exhibits most of the muscarinic but not the nicotinic properties of acetylcholine.<sup>4</sup> It is not clear whether or not pilocarpine produces a discharge of epinephrine from the adrenal gland for there are conflicting reports.<sup>5-10</sup> The occurrence of such a discharge would influence our studies since epinephrine causes pronounced bronchodilation and counteracts the action of some parasympathomimetic drugs on the respiratory tract in asthmatic subjects.<sup>11</sup> In 1921 Alexander and Piddock<sup>12</sup> gave 3 mg. of pilocarpine subcutaneously to twenty asthmatic patients and in ten, asthma-like attacks were precipitated as a result of the injection.

## METHOD AND MATERIALS

Pilocarpine hydrochloride was administered intramuscularly or intravenously in doses of 1 to 5 mg. to a group of normal subjects and to a group of asthmatic patients. The reaction of the respiratory tract was measured chiefly by recording the vital capacity on a rapidly moving drum according to a method previously described.<sup>1</sup> The first group was composed of ten subjects who had no personal or family history of allergy and no signs or symptoms of allergic disease or bronchitis. In the second group were seventeen ambulatory patients with bronchial asthma. No attempt was made to classify the type of asthma but the majority of patients were young adults subject to attacks at any time during the year. For

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purposes of comparison they were divided into three groups, depending chiefly on the number and severity of the attacks. The majority of these subjects had been studied previously, had performed numerous vital capacity tests, and were well trained and very cooperative.

Pilocarpine hydrochloride\* was dissolved in sterile isotonic saline to a concentration of 10 mg per milliliter and placed in a sterile rubber capped bottle. The solution was replaced at monthly intervals and stored in a refrigerator when not in use.

### RESULTS AND COMMENT

*Normal Subjects*—In the group of ten normal subjects the intravenous injection of 2 to 5 mg of pilocarpine produced no significant reduction in the vital capacity (Table I). Similar results were obtained after 6 mg doses of Mecholyl chloride given intramuscularly in a previous study.<sup>3</sup> There was no subjective sensation of tightness in the chest after pilocarpine, however, as

TABLE I EFFECT OF THE INTRAVENOUS ADMINISTRATION OF 2 TO 5 MG OF PILOCARPINE HYDROCHLORIDE ON THE VITAL CAPACITY IN TEN NORMAL SUBJECTS

SUBJECT	AGE (YR)	SEX	PILOCARPINE (IV, MG)	VITAL CAPACITY	
				BEFORE DPLG (ml)	CHANGE (%)
T H	32	M	2	4441	- 4
			2	4107	-10
R C	29	M	3	3459	+ 5
L J	25	M	3	5214	- 1
H O	23	M	2	4953	+ 5
S L	30	M	2	4577	+ 0.2
			5	4452	- 0.4
J C	32	M	2	4201	+ 2
			5	4107	+ 4
S K	30	M	3	4389	- 0.5
			5	4452	4
J S	26	M	3	3563	+12
R F	25	M	3	4765	- 0.2
P K	32	M	3	4065	+ 1

noted following the administration of Mecholyl chloride. Coughing was experienced occasionally and salivation persisted for a longer period of time after pilocarpine than after Mecholyl chloride. The increase in vital capacity after pilocarpine in some of the subjects perhaps might have been due to the fact that they were not so well trained as the asthmatic group. However, the control vital capacities were uniform. The sweating response in this group following comparable doses of pilocarpine was apparently uniform as judged by clinical observation. The heart rate was consistently elevated. In these respects the findings were similar to those obtained with Mecholyl.

*Asthmatic Subjects*—All of the seventeen asthmatic patients suffered a reduction in vital capacity after the administration of 1 to 4 mg of pilocarpine intramuscularly or intravenously and in many instances the reaction was severe (Table II). However, one subject (J B), who had mild asthma, had a reduction of only 2 per cent with 4 mg by vein, and another subject (R B), who had

\*Supplied by Merck and Company, Inc., Rahway, N. J.



TABLE II EFFECT OF THE INTRAMUSCULAR OR INTRAVENOUS ADMINISTRATION OF 1 TO 4 MG OF PILOCARPINE ON THE VITAL CAPACITY IN SEVENTEEN PATIENTS WITH ASTHMA COMPARED WITH THE EFFECT OF MECHOLYL CHLORIDE

SUBJECT	AGE (YR.)	SEX	SEVERITY OF ASTHMA			DRUG ADMINISTERED			VITAL CAPACITY	
						PILOCARPINE		MECHOLYL CHLORIDE (I M MG)	BEFORE DRUG (ML.)	CHANGE (%)
			1+	2+	3+	I M (MG)	I V			
F G	29	M		x		3			3469	-16
							1		3647	-17
								4	4159	-61
R C	42	F		x			1		2477	-27
W J	14	M		x			1		2456	- 8
								2	2278	-37
L S	23	F		x			2		2707	-22
								4	2257	30
T B	15	M	x				4		3250	2
								4	3156	-29
R B	25	F		x			2		2424	- 1
								2	2404	48
R B	19	M		x			2		4734	-22
								2	2989	47
M M	38	F		x			1		3093	5
								4	3396	18
H H	49	F		x			1		2414	-13
								1	2247	21
B S	13	F		x			2		1996	81
								2	1954	-69
W N	23	F		x			2		2738	29
								2	2613	-77
A L	33	M		x			2		3584	12
								4	4504	45
H W	31	F		x			2		2801	-46
								4	2424	70
W F	27	M	x				2		5225	5
								6	4681	-31
J D	16	F		x			2		2957	- 9
								1	3103	-46
F W	45	F		x		3			2853	-10
							4		2560	35
								2	2529	36
W B	47	M		x		3			3145	-16
							3		3010	-33
								2	2748	28

1+ Asthma patients with only a past history of asthma or with one or two attacks a year  
 + asthma patients with asthmatic paroxysms one or two times a month 3+ asthma patients  
 with one or two attacks or more a week

daily asthmatic paroxysms, had a reduction in vital capacity of only 1 per cent with an intravenous dose of 2 milligrams. The latter subject also experienced a reduction in vital capacity of only 3 per cent after histamine but in contrast the intramuscular administration of 2 mg of Mecholyl chloride was followed by a decrease in vital capacity of 48 per cent from the resting level of 2,404 milliliters. All the asthmatic patients given up to 6 mg of Mecholyl chloride in a previous study also reacted with a reduction in vital capacity. The intensity of the asthma like attack and the degree of reduction in vital capacity were greater after Mecholyl chloride than after pilocarpine with the doses used in these studies.

The degree of flushing, salivation, and sweating in the asthmatic patient did not appear to differ from the responses seen in the normal subjects after comparable doses of pilocarpine. In this respect our findings differed from those of Alexander and Paddock,<sup>12</sup> who concluded that flushing, salivation, and sweating were somewhat more pronounced in asthmatic patients than in normal subjects with similar doses of pilocarpine. The reaction pattern of the tracheobronchial tree, as determined by repeated vital capacity tests at intervals after the injection of pilocarpine, was variable, in contrast to the reaction after histamine and Mecholyl.

*Intramuscular Injection*—After the intramuscular injection of 3 to 4 m<sup>g</sup> of pilocarpine in the asthmatic patients, flushing, salivation, and mild sweating appeared within a few minutes, but the maximum reduction in vital capacity did not occur for from six to twenty-five minutes. The following case illustrates the reaction of Subject F G (Fig 1) to the intramuscular injection of 3 m<sup>g</sup>

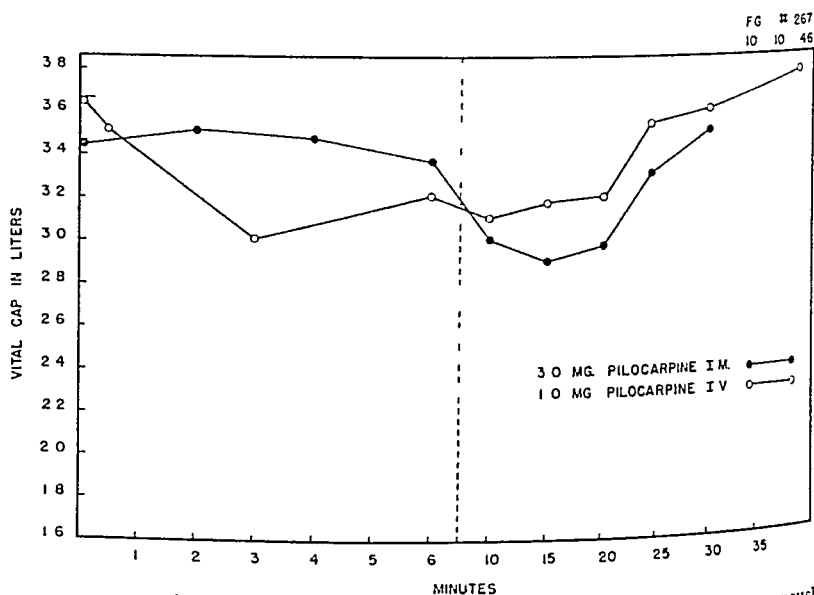


Fig 1—Comparative effect on the vital capacity of pilocarpine given intravenously and intramuscularly in Subject F G. Horizontal line represents time in minutes after the injection. Interrupted vertical line indicates change from one- to five-minute time intervals. The unshaded box on the left shows the control range of vital capacity determinations.

of pilocarpine. In the first six minutes after the injection, despite the subjective sensation of tightness in the chest, there was no reduction in the vital capacity. However, fifteen minutes following the pilocarpine administration there was a reduction of 543 ml in the vital capacity and wheezing appeared. The reaction to the drug had disappeared in thirty minutes and the vital capacity was near the resting level. The delay in appearance of tightness in the chest and the more prolonged delay in reduction in the vital capacity following the injection of pilocarpine clearly is not due to slow absorption, since other systemic effects such as salivation and flushing are manifest within a few minutes after administration of the drug. In this respect the reaction of the

tracheobronchial tree after intramuscular pilocarpine differs from the reaction due to intramuscular Mecholyl chloride. After the latter a notable reduction in vital capacity is present in two minutes and the maximal effect is present in four to six minutes. In many cases the vital capacity may have returned nearly to the resting level fifteen minutes after the intramuscular administration of the drug. It is interesting to note that the reduction in vital capacity in asthmatic subjects due to intramuscular or intravenous Prostigmine methylsulfate<sup>13</sup> is similarly delayed in onset and resembles the tracheobronchial reaction due to intramuscular pilocarpine. As will be shown later a second and delayed reduction in vital capacity was also found in many of our subjects after the intravenous administration of pilocarpine. The precise explanation for this delay of tracheobronchial reaction to pilocarpine is not clear. It is possible that the liberation of epinephrine as a result of the injection may in some way manifest a temporary protecting action against the tracheobronchial effect of the drug. It will be shown subsequently that epinephrine may afford protection against the effect of pilocarpine in the lungs without affecting the flushing, salivation, and sweating that result from its administration. Further studies are necessary to explain these findings.

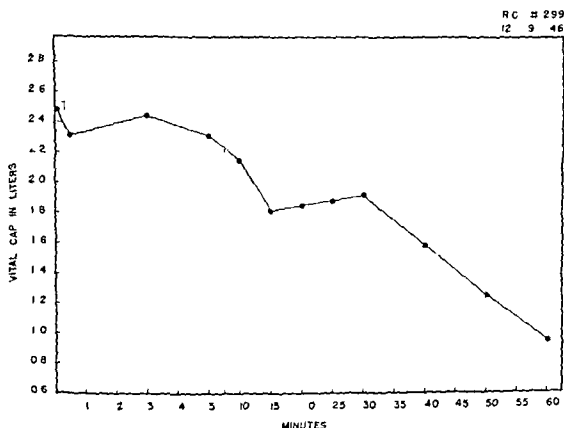


Fig. 1.—Changes in the vital capacity during the precipitation of an asthmatic attack in Subject R. C. with 1.0 mg. of pilocarpine given intravenously. Horizontal line represents time in minutes after the injection. Interrupted vertical line indicates change from one to five minute time intervals.

**Intravenous Injection**—After the intravenous administration of 1 to 4 mg. of pilocarpine the greatest reduction in vital capacity in most of the patients with asthma occurred in thirty seconds. In a few cases there was a greater reduction three minutes after the administration of the drug. In many cases, after a well established trend in the vital capacity toward the resting levels,

there was an evanescent secondary decrease in vital capacity ten to fifteen minutes after injection. This second reduction, although small, appeared to be a result of the pilocarpine injection since the subjects were well trained and such variations were not ordinarily experienced after histamine and mecholyl. The decrease in vital capacity varied in amount and was never of the same degree as the initial response to the drug. Even in the same subject it varied with successive identical doses of pilocarpine and did not always occur.

In one patient (R C) (Fig 2) a dose of 1 mg of pilocarpine produced a notable reduction in vital capacity, and when a definite trend toward normal, together with subjective improvement, had been established, an asthmatic attack occurred. A marked, further decrease in vital capacity resulted. This attack was readily alleviated by inhalation of aerosolized Isuprel 1-(3', 4' dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride\*.

The decrease in vital capacity following the intravenous injection of pilocarpine was greater than that due to intramuscular administration of an equal dose of the drug, but the ratio was variable. In Subject F W, 3 mg of pilocarpine injected intramuscularly produced a reduction in vital capacity of 9 per cent, or 282 ml, whereas an identical dose given intravenously caused a decrease of 18 per cent, or 501 milliliters. On another occasion 4 mg of pilocarpine were given intramuscularly and the vital capacity decreased 8 per cent, or 198 ml, whereas a similar dose by vein caused a reduction of 35 per cent, or 999 milliliters. In another subject (W B) 3 mg of pilocarpine injected intramuscularly reduced the vital capacity 9 per cent, or 292 ml, whereas the same dose given intravenously caused a decrease of 33 per cent, or 1,004 milliliters. In comparison with these studies the reaction of the respiratory tract to Mecholyl chloride given intravenously appeared to be ten to fifty times greater than an identical dose given by the intramuscular route.

In four subjects, 0.5 mg of Prostigmine methylsulfate was administered fifteen to thirty minutes prior to the injection of pilocarpine. In two instances it appeared to augment the action of pilocarpine on the tracheobronchial tree. For example, in Subject J D 2 mg of pilocarpine given intravenously produced a reduction of 291 ml in the vital capacity from the resting level of 2,957 milliliters. When the level had returned toward normal, 0.5 mg Prostigmine was injected by vein and the vital capacity fourteen minutes later measured 2,604 ml, a reduction due to Prostigmine itself of 303 milliliters. A dose of 2 mg of pilocarpine was repeated one minute later and a further reduction of 428 ml was noted. The systemic effects of pilocarpine—namely, flushing, salivation and sweating—also appeared to be augmented by the action of Prostigmine. In two instances Prostigmine did not appear to augment either the tracheobronchial or systemic effects of pilocarpine.

When repeated identical doses of pilocarpine were administered at thirty-minute intervals to five subjects, variable results were obtained. In two subjects similar reductions in vital capacity occurred, but in the other three it appeared that with successive doses there was a markedly diminished effect of

\*Furnished by Frederick Stearns & Co. Detroit, Mich.

the drug in reducing the vital capacity. For example, in Subject B S (Fig 3) the intravenous injection of 2 mg of pilocarpine caused a reduction of vital capacity to 376 ml from a resting level of 1,996 milliliters. A second injection forty five minutes later produced a reduction to 993 ml and a third dose thirty minutes later reduced the vital capacity only to 1,379 milliliters. The

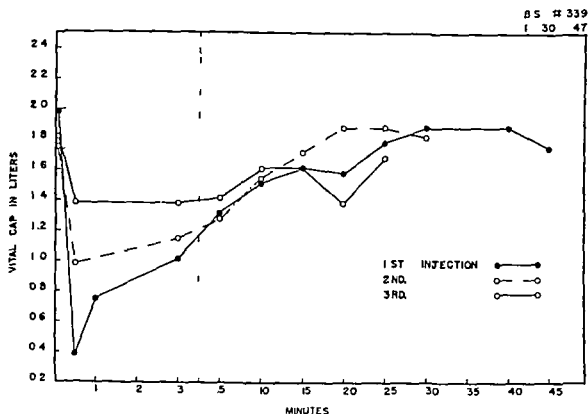


Fig 3—Comparative effect on the vital capacity of three 0.0 mg doses of pilocarpine injected intravenously at intervals. Horizontal line represents time in minutes after the injection. Interrupted vertical line indicates change from one to five minute time intervals.

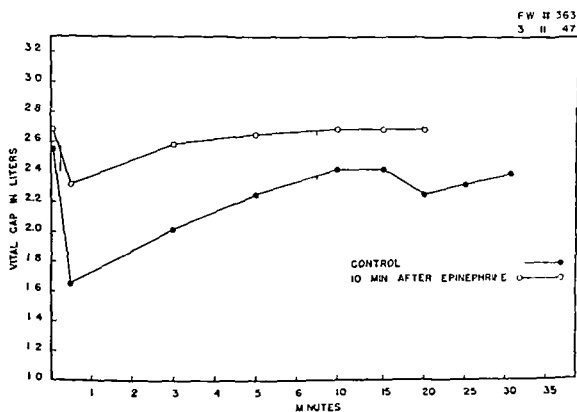


Fig 4—The effectiveness of 0.1 mg epinephrine given intramuscularly in blocking the reduction in vital capacity caused by the intravenous injection of 4.0 mg of pilocarpine. The horizontal line represents time in minutes after the injection. The interrupted vertical line indicates a change from one to five minute time intervals.

subjective sensation of tightness in the chest also became less with repeated injections. In another subject (F G) a similar trend resulted after four successive injections at thirty-minute intervals, so that the final injection produced no reduction in vital capacity. When the experiment was repeated several weeks later, however, identical doses of pilocarpine produced similar reductions in vital capacity.

Certain antiasthmatic drugs afforded protection in the reactive subjects against the reduction in vital capacity due to pilocarpine. In two cases epinephrine gave pronounced protection against this reduction. Patient F W (Fig 4), for example, had a resting vital capacity of 2,560 ml, and thirty seconds after the intravenous administration of 4 mg pilocarpine it measured 1,651 milliliters. After a gradual return toward normal, 0.3 ml of 1:1,000 epinephrine was injected intramuscularly and five minutes later the vital capacity was 2,686 milliliters. A repeat injection of 4 mg of pilocarpine produced a reduction in vital capacity to only 2,330 ml, and there was a rapid return to the resting level. It was also noted that although epinephrine appeared to prevent the reduction in vital capacity and the subjective and objective changes in the lungs following the administration of pilocarpine, it did not appreciably affect the salivation, flushing, and perspiration resulting from the injection of the drug. In Patient H W epinephrine not only afforded complete protection against the reduction in vital capacity due to pilocarpine but also appeared to reverse the action of the drug. Following the repeat injection of 2 mg of pilocarpine there was an increase of 460 ml in the vital capacity, compared with a decrease of 1,098 ml caused by the same amount of pilocarpine prior to the administration of epinephrine.

Theophylline ethylenediamine likewise prevented the reduction in vital capacity due to pilocarpine. In Subject F G the resting vital capacity measured 3,731 ml, and thirty seconds after the intravenous injection of 3 mg of pilocarpine it measured 2,727 milliliters. When the vital capacity returned to the resting level, 350 mg of theophylline ethylenediamine were given slowly by vein, and ten minutes later the vital capacity was 3,760 milliliters. A second dose of 3 mg of pilocarpine then produced a reduction in vital capacity to only 3,647 milliliters. The systemic reaction to pilocarpine was not altered appreciably.

Bellafoline,\* a stable preparation of total levorotatory alkaloids of belladonna leaves, likewise afforded protection against the reduction in vital capacity due to pilocarpine. Patient R C, who had previously suffered an acute asthmatic attack following the injection by vein of 1 mg pilocarpine, was given 0.5 mg of Bellafoline intramuscularly. Ten minutes later an intramuscular dose of 30 mg of pilocarpine produced a decrease in vital capacity of only 53 milliliters. The degree of protection thus afforded the tracheobronchial tree by epinephrine, theophylline ethylenediamine, and Bellafoline was similar to the protection given by the same drugs against Meecholy chloride.<sup>11</sup> The other reactions to pilocarpine, such as sweating, flushing and salivation, were only slightly affected by the protecting drugs.

\*Furnished by Sandoz Chemical Works Inc. New York N Y

It appears that attempts to assay the effectiveness of various anticholinergic drugs by their capacity to prevent the asthma like attacks and the reduction in the vital capacity induced by cholinergic substances would be better carried out with Meecholy chloride than with pilocarpine as the test substance, since the former drug has a more uniform action. Since anticholinergic agents such as atropine and Bellafoline are very effective in preventing this type of induced asthma it would seem worth while to reinvestigate their use in the treatment of spontaneous asthma, especially since other drugs in this group are available which do not have the unfavorable side reactions of atropine. More support for such an investigation is derived from recent data which indicate that in the asthmatic subject the pulmonary reaction to Meecholy chloride may be greater and perhaps more important than the reaction to histamine.<sup>3</sup> Confirmation of the pulmonary hyperresponsiveness of asthmatic subjects to cholinergic drugs also suggests that this manner of study should be carried out when surgery on the autonomic nervous supply to the lung is contemplated. It is possible that those subjects who exhibit a more pronounced reactivity to the cholinergic drugs may benefit most by resection of the posterior pulmonary plexus.

#### SUMMARY

The reaction of the respiratory tract to doses of 1 to 5 mg of pilocarpine in a group of ten normal subjects and seventeen patients with asthma was studied chiefly by measurements of the vital capacity. In two of the normal subjects there was a slight decrease in vital capacity after pilocarpine. In contrast, all the asthmatic patients suffered a reduction in vital capacity after the administration of 1 to 4 mg of pilocarpine intramuscularly or intravenously. After the intramuscular injection of pilocarpine the period of greatest reduction in vital capacity occurred in six to twenty five minutes whereas flushing, salivation, and sweating occurred in a considerably shorter time. The greatest reduction in vital capacity with intravenous pilocarpine usually occurred in thirty seconds, but there was frequently a second reduction ten to fifteen minutes after the injection. Prostigmine appeared to augment the action of pilocarpine in two of four subjects. Epinephrine, theophylline, ethylenediamine and Bellafoline protected the tracheobronchial tree against the reduction in vital capacity due to pilocarpine, but failed to have much effect on the flushing, salivation and sweating due to the drug.

Clinical implications of the study are discussed.

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A COMPARISON OF THE TOXIC MANIFESTATIONS PRODUCED BY  
BETA DIMETHYLAMINOETHYL BENZHYDRYL ETHER HYDRO  
CHLORIDE (BENADRYL) AND TRIPLENNAMINE  
(PYRIBENZAMINE)

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THE therapeutic value of antihistaminic compounds in many allergic conditions seems now to be well established. Of these compounds, Benadryl (beta dimethylaminoethyl benzhydryl ether hydrochloride) and Pyribenzamine (N' pyridyl N' benzyl N dimethylethylenediamine) have had by far the most extensive clinical trial. There are a number of other preparations which either in laboratory animals or in man or in both have shown antihistaminic activity equal to or greater than either of these new drugs.<sup>1, 2</sup> Nevertheless until such materials are more thoroughly tested in the human being we must rely mainly upon the two drugs first mentioned whenever an antihistaminic agent is required.

Any comparison of these two substances in human beings is fraught with several difficulties. In the first place the results in animals cannot be transferred quantitatively, and probably not qualitatively to human beings even in regard to the ability of these substances to prevent death from histamine aerosols or anaphylactic shock. Moreover the alkamine ether and tripeleennamine differ in several particulars pharmacologically. The former possesses marked anticholinergic and antispasmodic activity; the latter has little or none.<sup>1, 3</sup> Benadryl acts oppositely to tripeleennamine upon the musculature of the duodenum and the uterus. In the human being both exert some hyoscine like action which is more marked with Benadryl.<sup>4, 5</sup>

Comparisons of the therapeutic efficacy of Benadryl and Pyribenzamine are few in number.<sup>3, 6, 11</sup> Claims have been made that dose for dose the drugs are equally effective,<sup>6, 10, 11</sup> or that Benadryl<sup>8</sup> or tripeleennamine<sup>3, 9</sup> is more powerful. One worker who found the latter more active than the former<sup>3</sup> has failed to mention the doses used in the comparisons although by inference he has left the impression that that employed for Benadryl was often if not usually, one half to one third that of tripeleennamine. The second investigator, who more consistently observed better effects in some clinical conditions from tripeleennamine, states that the doses of the latter varied from 200 to 400 mg. daily while those for the alkamine ether were usually 200 mg. daily.<sup>9</sup> Other workers<sup>6, 8</sup> made their comparisons on a weight basis.

There is rather general agreement regarding the nature of the side effects following the use of both Benadryl and tripeleennamine, but there is marked difference of opinion concerning their incidence and severity. To date, all or

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nearly all of the observations regarding these points have been recorded in the course of treatment of allergic diseases and are therefore subject secondarily to all the influences surrounding the allergic episode. Moreover, there has been no concerted effort to compare the reactions of both drugs in the same subject. Thus the personal equation of the subject has never been eliminated from the final evaluation of the results. In an effort to accomplish these objectives and to afford a sound basis for the comparison of the toxic activity of these antihistaminic compounds over the entire range of therapeutic dosage, the present study was undertaken.

#### MATERIALS AND METHODS

Ambulatory patients from a general medical clinic of a city hospital were chosen at random except that those with acute illnesses, allergic complaints, or recognizable disturbances of the autonomic nervous system were excluded. The underlying conditions for which these patients sought relief included arthritis, hypertension, arteriosclerosis, and minor upper respiratory, muscular, or digestive complaints. Each subject was further tested for his or her fitness to participate in the investigation by the use of a placebo which resembled the corresponding antihistaminic compound in all its physical characteristics.\*

In all, one hundred forty-two subjects, eighty women and sixty-two men ranging in age from 18 to 80 with an average of 41.7 years, completed the test. Of these, fifty-two were studied while taking Benadryl only, fifty-two while receiving tripeleminamine only, and thirty-eight while ingesting successively each of the two antihistaminic compounds. In the last-mentioned subjects a period of fourteen days or more elapsed after one drug had been used before the second was started. Each drug was tried for not less than one week, and any given level of dosage was continued in each subject for not less than one week. A number of subjects were given gradually increasing doses of one or the other drug. The patient continued at each level of dosage for at least one week. Therefore some patients received one or the other of the antihistaminic compounds for periods lasting from four to eight weeks. However, gradually increasing doses of the drug were avoided in the majority of instances, as there was thus produced a tendency to build a tolerance against the unpleasant reactions to the compound.

In order to establish a relatively long-term comparison between the two drugs, each of six subjects was successively followed on gradually increasing doses of first one drug and then the other at daily levels of 150, 300, 450, and 600 mg respectively. In these subjects each increment in the amount of compound ingested was made after a period of not less than seven days at the immediately preceding level of dosage. Two weeks or more elapsed following the use of one preparation before the other was started.

In Tables I and II the number of patients who developed toxic reactions at each level of dosage and the nature and frequency of various types of reaction

\*We wish to express our appreciation to Dr. E. A. Sharp of Pike Drug Co., Detroit, Mich., for generous supplies of Benadryl and placebo and to Dr. R. D. Moyer of Ciba Pharmaceutical Products, Inc., Summit, N. J., for a portion of the tripeleminamine.

TABLE I THE NATURE AND INCIDENCE OF TOXIC REACTIONS TO BENADRYL AND PYRIBENZAMINE IN RELATION TO DOSAGE OF COMPOUND USED

DAILY DOSE (MG)	CASES			INCIDENCE AND TYPES OF REACTION											
	TOTAL NUMBER	WITH REACTION		DROWSINESS	DRYNESS OF MOUTH	DIZZINESS	WEAKNESS	NAUSEA AND EPIDIDYMIC DISTRESS	VOMITING	CONSTIPATION	SHALLOENESS OF BREATHING	HEADACHE	ANOREXIA	SLEECH AND VISUAL DISTURBANCES	DIAPHRAGM
		NUMBER	PERCENTAGE												
Benadryl															
150	14	9	64	8	2	4		1							
300	14	11	79	7	3	2	1						3		
450	13	10	76	7	2	6	2	1		1		1			
600	11	10	91	8	6	4	6	4	1	1	2		1	2	
Total	52	40	77	30	16	16	9	6	1	2	5	1	4	2	
Pyribenzamine															
150	14	9	64	6	5	2		2		1		2	1	1	1
300	14	9	64	5	2	1	1	1				2	1		1
450	13	11	73	7	9	2	1	3		1	1		2		
600	9	6	66	3	6	1	1	2		2	1	2			
Total	52	35	67	21	25	6	3	8	0	2	3	5	6	1	2

Here are recorded results in 104 patients one half of whom received Benadryl and the other half triptelennamine

are recorded. It will be noted that at the usually employed daily therapeutic dose 150 mg, the incidence of side effects was approximately the same for both drugs. The actual percentages are rather high as contrasted with previously reported results. However since the primary aim was to recognize all unpleasant symptoms rather than to determine therapeutic efficacy a record was made of each manifestation even though present in minor degree.

TABLE II THE INCIDENCE OF UNDESIRABLE EFFECTS IN THIRTY EIGHT SUBJECTS WHO RECEIVED IDENTICAL COURSES OF TREATMENT WITH BENADRYL AND PYRIBENZAMINE, RESPECTIVELY

NATURE OF MANIFESTATION	NUMBER OF PATIENTS SHOWING SYMPTOMS AT THE LEVELS OF DOSAGE INDICATED									
	11 150 MG		12 300 MG		9 450 MG		6 600 MG		TOTAL 38	
	B*	P†	B	P	B	P	B	P	B	P
Drowsiness	7	4	5	3	5	3	6	3	23	13
Dryness of mouth	1	5	3	5	3	6	2	5	10	11
Dizziness	3	2	2	1	4	1	2	1	11	6
Gastrointestinal disturbances	1	3	0	1	1	2	3	1	6	7
Headaches	0	2	0	1	0	0	0	1	0	4
Anorexia	0	1	1	2	0	2	0	1	1	6
Palpitation	0	0	0	1	0	0	0	0	0	1
Tinnitus aurium	0	1	0	0	0	0	0	0	0	0
Weakness	0	0	0	1	1	1	3	0	4	2
Visual disturbances	0	0	0	1	0	0	1	0	1	1
Shakiness or jumping	0	0	0	0	0	0	0	1	3	1
Total	13	18	11	11	15	18	10	13	40	63

B Benadryl.

† Pyribenzamine or triptelennamine

Of the group of fifty-two subjects who received Benadryl, 77 per cent of the total exhibited some evidence of toxicity, and among the fifty two persons given tripeleennamine only, 67 per cent developed undesirable reactions. An analysis of the results obtained with the thirty-eight individuals who received corresponding doses of both drugs for identical periods of time (Table II, Fig 1) confirmed the findings obtained when entirely different groups of subjects were employed for the testing (Table I). Six subjects, in whom each of the drugs was employed in daily doses of 150, 300, 450, and 600 mg, respectively, for a period of one week or more, did not develop any manifestations of toxicity which persisted or became chronic following the discontinuance of either or both drugs. It was definitely demonstrated that with small doses of each drug, signs

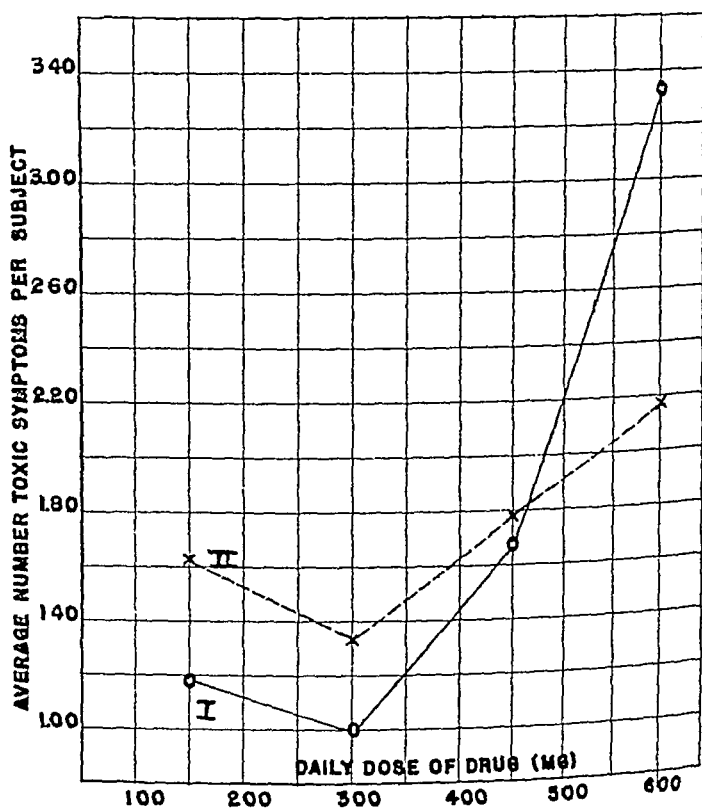


Fig 1—Toxic symptoms produced in a group of thirty eight subjects following the oral administration of Benadryl (I) and Pyribenzamine (II) respectively for periods of one week or longer at each of the dosage levels indicated

and symptoms of toxicity were frequent, but mild in degree, both drugs were well tolerated. Drowsiness was more common in connection with the administration of Benadryl, and dryness of the mouth, with tripeleennamine. With Benadryl, a tolerance against drowsiness usually developed in a few days, except with the use of the largest doses when cerebral manifestations became severe. When tripeleennamine was used dryness of the mouth was common and increased in intensity as the daily dose was augmented. Regardless of the amount of drug

employed within the limits noted the majority of the other side reactions to tripeleminamine, such as drowsiness, remained approximately the same. In other words, the tendency to establish tolerance was less than with Benadryl.

#### DISCUSSION

Both experimentally and clinically tripeleminamine and Benadryl have been demonstrated to possess qualities powerfully antagonistic to histamine. Upon this common feature much of their efficacy in the treatment of allergic disease appears to rest. In addition to this Benadryl shows a minor but still powerful hyoscyne-like action not fully shared by Pyribenzamine. This provides in addition to the antihistaminic effect a mild sedative action which is often desirable in the control of many hypersensitive reactions.

Opportunity has been afforded to utilize human subjects for determining the comparative toxicity of Benadryl and tripeleminamine. The facts that a representative number of these individuals received each of the two drugs at different times and that all observations were checked by the same techniques and personnel lend added emphasis to certain points brought out by an analysis of the data.

1 The high incidence of side effects. All side effects were recorded regardless of their severity. When lower levels of dosage (150 and 300 mg) were used side effects rarely precluded the continued use of the drug. However their frequency prompts a continued search for more nearly ideal agents in the management of the allergic reactions due to histamine.

2 The nature and percentage of side effects produced by each of the two drugs. It is clear from the present study that the intensity and number of cerebral manifestations is greater with Benadryl than with Pyribenzamine. It is equally evident that symptoms referable to the gastrointestinal tract are much more frequent with tripeleminamine than with Benadryl. When the overall incidence of all reactions is assessed there is little difference between the two drugs at lower levels of dosage that is 150 to 300 mg daily. Indeed the greater drowsiness caused by Benadryl is often of therapeutic importance particularly in the itching dermatoses and in asthma. However when higher levels of dosage (450 to 600 mg) are employed Benadryl is decidedly the more toxic of the two drugs (Fig 1) and may cause confusional states not unlike those seen following alcoholic excesses.

From the present studies it seems that far too much emphasis has been placed upon the relatively high incidence of reactions to Benadryl for instance 75 per cent,<sup>2</sup> as compared with the infrequency of reactions to Pyribenzamine for instance 25 per cent or less.<sup>2</sup> Indeed in the usual range of therapeutic doses (from 150 to 300 mg daily) a critical study shows that this wide difference does not exist. Moreover, many of the unpleasant symptoms produced by Benadryl in such amounts are actually accounted for by its sedative action, which may sometimes be profitably employed in therapy. At higher levels of dosage Benadryl becomes definitely the more toxic of the two antihistaminic substances under

discussion (Fig. 1) and should be used at such levels only when the patient can be under more or less constant surveillance. The gastrointestinal irritation of tripeleminamine at similarly high levels of dosage usually precludes its use in the majority of patients.

3. Is there a direct relationship between toxic reactions and the antihistaminic effect? If this question is answered in the affirmative, one would expect that therapeutic effectiveness and toxicity would vary directly. However this is not the case. It seems likely, therefore, that the antihistaminic factor is not responsible for the untoward symptoms, but that some other property of each of these drugs plays a dominant role in producing side effects. This apparent dissociation between antihistaminic and toxic properties of these drugs furnishes a real incentive to continue the search for agents which will possess the antihistaminic factor to the complete or nearly complete exclusion of the toxic factor.

#### SUMMARY AND CONCLUSIONS

One hundred forty-two subjects without allergic disease or known disturbances of the autonomic nervous system were given Benadryl and tripeleminamine (Pyribenzamine), respectively, in doses ranging from 150 to 600 mg daily.

Of 52 subjects to whom tripeleminamine alone was given, thirty-five, or 67 per cent, developed one or more toxic symptoms. Of an equal number of subjects to whom Benadryl alone was administered, forty, or 77 per cent, showed some type of untoward reaction.

In thirty-eight patients the effects of both Benadryl and tripeleminamine were determined and comparisons of the effects of the two drugs were made on a weight for weight basis. The total number of reactions to Benadryl was sixty, and to tripeleminamine, sixty-three. When 450 mg or less of the drug were given, Benadryl showed definitely fewer reactions than did Pyribenzamine, but above that figure Benadryl produced a decidedly higher incidence of reactions.

Benadryl produced a preponderance of its disturbances in the sensorium, tripeleminamine showed a predominance of gastrointestinal manifestations.

Usually the side effects did not preclude the therapeutic application of either drug in daily doses of 300 mg or less. With Benadryl, initially unresponsive responses may disappear after the drug is continued for several days.

Both drugs are highly useful antihistaminic substances in the control—of a number of allergic diseases. In the usual range of dosage the incidence of side reactions is approximately equal and their intensity so mild as rarely to preclude their continued use. Indeed, the cerebral depression caused by both drugs, but more especially by Benadryl, may frequently add to the range of therapeutic applicability.

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## EVALUATION OF A NEW SEDATIVE DRUG (3,3-DIETHYL-2,4-DIOXOPIPERIDINE)

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MOST physicians are confronted daily with the need of sedative medication for many of their patients. The value of the bromides and barbiturates is firmly established. However, these drugs occasionally produce side reactions such as rashes, headache, and lassitude. Moreover, continued use of the barbiturates may lead to tolerance or addiction. In the light of the foregoing, a new sedative which is distinguished by clinical effectiveness and good tolerability deserves consideration. Consequently, we set out to evaluate such a compound (3,3-diethyl-2,4-dioxopiperidine) submitted to us under the designation of NU-1510 and later under the name of Sedulon.\*

The pharmacology of this substance and its oxidation product, 3,3-diethyl-2,4-dioxotetrahydropyridine, was thoroughly explored by Koppányi, Herwult, Linegar, and Foster.<sup>3</sup> According to these investigators both compounds, in appropriate doses, produce motor paralysis, muscular relaxation, loss of righting reflexes, and deep sleep in experimental animals. The onset of action with the piperidine derivative is somewhat slower and its effect weaker and longer lasting than with the pyridine compound. From the figures presented by these workers it appears that, administered intravenously to rabbits, the narcotic index ( $LD_{50}/ND_{50}$ )† of the piperidine derivative is 3.42, that of the pyridine compound, 4.07, and that of barbitol, 2.96. Thus, the relative order of safety was found to be pyridine derivative > piperidine compound > barbitol. There were no significant changes in hemoglobin, erythrocyte count, leucocyte count, or differential count when daily doses of the pyridine derivative and the piperidine compound, respectively, were fed to rabbits for several weeks.

3,3-Diethyl-2,4-dioxotetrahydropyridine was evaluated clinically by Polatin and Horwitz<sup>7</sup> in psychiatric cases and by Friedl<sup>1</sup> in mentally normal patients requiring sedative-hypnotic medication. These workers found the pyridine derivative to be a mild but very effective somnifacient. As a daytime sedative it generally proved adequate in patients with nervous tension.

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† $LD_{50}$  is defined as the lethal dose for 50 per cent of the animals and  $ND_{50}$  as the narcotic dose which prevents a righting response in 50 per cent of the animals after the tail are pinched.



Because of its weaker and more prolonged action as evidenced in animal experiments, 3,3 diethyl 2,4-dioxopiperidine held promise of being at least as satisfactory as its oxidation product for daytime sedation and it was in this direction that a study was instituted.

The clinical work is presented in Part I which gives results in patients requiring sedative medication. Part II gives the work done in man on urinary excretion following the administration of 3,3 diethyl 2,4 dioxotetrahydropyridine and of 3,3 diethyl 2,4 dioxopiperidine.

## PART I

BY AARON E. PARSONNET, ARTHUR BERNSTEIN AND EMANUEL KLOSK

The initial program called for an intensive study of a small group of subjects. Twelve patients with a diagnosis of essential hypertension were chosen. There were five men, ranging in age from 49 to 64 years, and seven women whose ages ranged from 27 to 56 years. Some of these patients had cardiac symptoms such as angina of effort, dyspnea and palpitation; others complained merely of the frequent headaches and nervous manifestations so often endured by the hypertensive patient. All of them had been taking  $\frac{1}{4}$  to  $\frac{1}{2}$  gram of phenobarbital three or four times a day for periods ranging from several months to a number of years and were thus suited for a comparative study.

Phenobarbital and all other medication were discontinued. Sedulon was substituted and the original plan of studying these subjects over a period of approximately two months was followed with but minor deviations. It was explained to each patient that he was going to receive a drug which might have an effect on the blood pressure but no mention was made of sedative action. Deliberately their attention was diverted and all questions related to the sedation attained were injected casually in the course of subsequent interviews. It was thought that there was no need for alternating Sedulon and placebo medication since phenobarbital, known to the patients as exerting a sedative effect, was replaced by a drug which they expected to have a hypotensive but not a sedative action. Consequently any results in the latter direction were probably not conditioned by a psychologic influence.

Treatment of eleven subjects was instituted with doses of 0.125 Gm. but only in one did this amount suffice to induce good sedation. The other ten subjects complained of nervous tension on a total daily dose of 0.375 Gm. and this was therefore doubled. However the increase was not ordered before these patients had been on the lower dose for from three to four weeks and the change was effected always at an interview when there was no complaint about inadequate sedation.

One patient did not return for re-examination after she was placed on 0.25 Gm. three times daily. Of the nine subjects who were followed further seven derived good sedation from a total daily dose of 0.75 Gm. and in two only fair sedation was induced. A twelfth patient received initially 0.25 Gm. three times a day and this was attended by satisfactory sedation.

The action of Sedulon commenced thirty to sixty minutes after its intake and lasted from three to four hours following each dose. The effect was found

to be of a selective sedative nature without marked soporific action. This quality is an advantage since doses of phenobarbital which were productive of a similar degree of sedation commonly rendered the patients sleepy at the same time.

The patient deriving good sedation from only 0.375 Gm. per day complained of pruritus, which was first thought to be due to the medication. With diavial of the drug, however, failed to result in subsidence of the pruritus which was finally relieved by psychotherapeutic measures. Reinstitution of Sedulon was not attended by recurrence of the pruritus. Similarly, there were no ill effects in any of the other patients.

Blood pressure readings for all subjects were done at frequent intervals. As can be seen from the average values in Table I, there was no significant reduction in either the systolic or the diastolic blood pressure.

TABLE I  
AVERAGE BLOOD PRESSURE READINGS AND HEMATOLOGIC FINDINGS IN TWELVE  
HYPERTENSIVE PATIENTS TREATED FOR FROM FOUR TO EIGHT WEEKS WITH  
3,3 DIETHYL 2,4 DIOXOPIPPIDINE (SEDULON)

	BEFORE TREATMENT	AFTER TREATMENT	RANGE OF WEEKLY AVERAGES	
			MINIMUM	MAXIMUM
Blood pressure	192/108	184/107	--	--
Hemoglobin (per cent) (Suhl)	91	96	91	95
Blood cell counts				
Erythrocytes (millions/c. mm.)	4.46	4.46	4.37	4.58
Leucocytes (millions/c. mm.)	7.64	8.13	7.04	9.13
Polymorphs (per cent)	59.4	61.8	54.0	66.1
Lymphocytes (per cent)	30.1	28.6	22.7	32.3
Monocytes (per cent)	7.4	7.4	6.5	9.0
Eosinophils (per cent)	2.7	2.6	2.6	3.0

Urinályses carried out every week did not reveal any changes as compared with the premedication findings.

A special effort was made to determine the effect, if any, of the prolonged treatment with Sedulon on the blood picture. Hemoglobin determinations and red, white, and differential counts were done for all patients initially and at weekly intervals. As can be seen from Table I, which lists the average findings before and after treatment as well as the range of weekly averages, there were no significant changes in the hemograms during this period of close observation which included eight blood counts for most of the patients.

These gratifying results prompted us to extend the initial program.

Sedulon was thus continued in eight of the original group of twelve patients (Table II). Including the initial trial period, these eight subjects remained on the experimental drug for from six months to almost one year. In Patient 4 a total daily dose of 0.375 Gm. continued to induce good sedation for nearly eleven months, that is, up to the time the patient last reported for reexamination. The other seven subjects, each of whom received 0.25 Gm. three times a day, continued to derive the same degree of sedation during this period of extended trial as they did initially, that is to say, in Patients 1, 7, 9, 10, 11, and 12, good sedation was induced, and fan sedation resulted in Patient 8. Ill effects were absent and none of the subjects developed signs of tolerance or addiction.

TABLE II BLOOD STUDIES IN EIGHT HYPERTENSIVE PATIENTS RECEIVING SEDULON FOR FROM SIX MONTHS TO ALMOST ONE YEAR

PATIENT	MEDICATION (MO.)	HEMOGLOBIN (PER CENT) (SAHLI)	ERYTHROCYTE COUNT (MILLIONS/C MM.)	LEUCOCYTE COUNT (THOUSANDS/C MM.)
1	0	92	4.40	6.40
	7 $\frac{1}{2}$	96	4.20	5.80
	9 $\frac{1}{2}$	102	5.00	10.20
4	0	100	4.47	6.30
	8	84	3.67	6.90
	10 $\frac{3}{4}$	87	3.80	6.20
7	0	90	4.40	6.30
	6 $\frac{1}{2}$	93	4.34	4.40
	10 $\frac{1}{4}$	88	4.08	4.80
	11 $\frac{1}{4}$	90	4.34	5.20
8	0	86	4.03	7.20
	7	100	4.80	14.10
9	0	104	5.23	7.30
	6 $\frac{1}{4}$	86	4.30	8.40
	7 $\frac{1}{4}$	84	4.34	9.40
	9 $\frac{3}{4}$	80	4.40	5.50
	10 $\frac{1}{2}$	94	4.41	6.40
10	0	90	4.09	6.00
	7 $\frac{1}{4}$	87	84	5.00
	10 $\frac{1}{4}$	93	4.35	
11	0	87	4.04	6.00
	5 $\frac{1}{4}$	86	4.13	7.30
	7 $\frac{1}{2}$	91	4.30	8.00
12	0	75	4.17	8.00
	6	81	4.30	6.20

0 The figures listed are premedication findings

During the period of extended trial hemograms were done for all eight patients at irregular intervals. Table II correlates some of the premedication blood findings and the findings after several months of medication. There occurred a decrease in the hemoglobin values and the erythrocyte counts in two patients (Patients 4 and 9) and a drop in hemoglobin values as well as in the red and white cell counts in one patient (Patient 7). However Patient 4 was on a rice diet at the time of the shift in the red blood picture and Patient 7 underwent a cholecystectomy in May 1947. In the former reinstitution of a normal diet was followed by an increase in both the hemoglobin value and the red cell count, and all the values of the last count recorded for Patient 7 at a time when she had completely recovered from the operation approximated the original findings. As for Patient 9 the erythrocyte count had dropped from 5,230,000 to 4,300,000 and the hemoglobin value had decreased from 104 to 86 per cent after more than six months of Sedulon intake. However there was no further reduction in the number of red blood cells during the ensuing four months in spite of continued Sedulon medication and the hemoglobin value increased appreciably during this time. It is concluded that none of these changes is attributable to the administration of the drug.

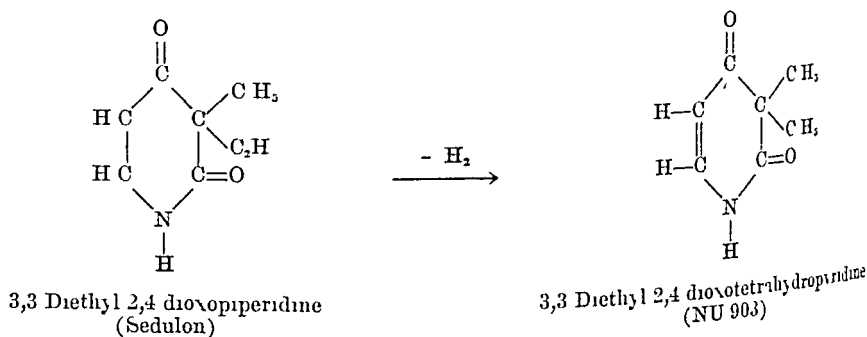
In further extension of the original program the preparation was given to fifty patients complaining of nervousness, irritability or emotional instability. In this group no blood studies were performed but each patient was seen individually. The dosage was 0.25 Gm. three times daily and most of these fifty

patients have been taking the drug for from three to five months. While good or fair sedation resulted from this regimen, generally the patients were not over-sedated. However, in one patient it became necessary to reduce the dosage to 0.125 Gm. three times a day, and this dose was attended by satisfactory sedation. One patient developed some dizziness and nausea after taking the drug for about one month. Twenty-four hours after discontinuation of the medication, these symptoms subsided and did not reappear when Sedulon was reinstituted forty-eight hours later. However, since this patient was apprehensive about continuing the drug, it was stopped several days thereafter. Among the fifty subjects of this group there was one patient who had previously developed dermatitis from the use of phenobarbital. In contrast, Sedulon, which she has been taking for three months, is tolerated without untoward effects.

## PART II

By ERICH HIRSCHBERG, SAUL H. RUBIN, AND LEO A. PIRK

As was shown by Krautwald and co-workers,<sup>5</sup> there are no indications that 3,3-diethyl-2,4-dioxopiperidine is excreted unchanged in the urine when this compound is fed to dogs at a dosage level of 200 mg. per kilogram. However, these workers did recover between 7 and 8 per cent of the amounts of the administered piperidine derivative in the form of its oxidation product—3,3-diethyl-2,4-dioxotetrahydropyridine. The following structural formulas illustrate this transformation of the piperidine into the pyridine compound:



With a new fluorimetric method<sup>2</sup> (a modification of that described by Kubli and Schmidt<sup>4</sup>) for the determination of this pyridine derivative in urine at our command, it was decided to study its renal elimination in man following the administration of 3,3-diethyl-2,4-dioxopiperidine. It seemed of particular interest to compare the amounts of the pyridine derivative excreted after the intake of the piperidine compound on the one hand, with those following the administration of similar doses of the pyridine compound on the other.\*

\*For the sake of simplicity, 3,3-diethyl-2,4-dioxopiperidine is referred to as Sedulon, and 3,3-diethyl-2,4-dioxotetrahydropyridine as NU-903, the designation under which the latter compound was supplied by Hoffmann-La Roche Inc., Nutley, N. J.

Four normal, male volunteers (physicians) ranging in age from 24 to 32 years were chosen. Collection of twenty-four hour urine specimens was instituted seven days prior to the initiation of the excretion experiment proper and continued daily for five weeks. On the eighth evening each subject received 0.6 Gm of NU 903 and after one week without further medication this dose was repeated. Again following a medication free interval of seven days 0.75 Gm of Sedulon was given to each of the volunteers and a second identical dose was given at the end of the fourth week with subsequent collection of urine specimens for another week.

Amber bottles containing approximately 5 cc of toluene were used for pooling the daily voidings of each subject. In carrying out the estimations of the urinary excretion of NU 903 in all the specimens thus obtained the method described by Hirschberg and co-workers<sup>2</sup> was used. This technique is based on the measurement of the comparative fluorescence of urines at suitable dilutions in the absence and presence of hydroxylamine. The difference in intensity between total fluorescence (without hydroxylamine treatment) and residual fluorescence (with hydroxylamine treatment) yields a measure of the concentration of Nu 903 in urine. Sedulon is nonfluorescent under these conditions.

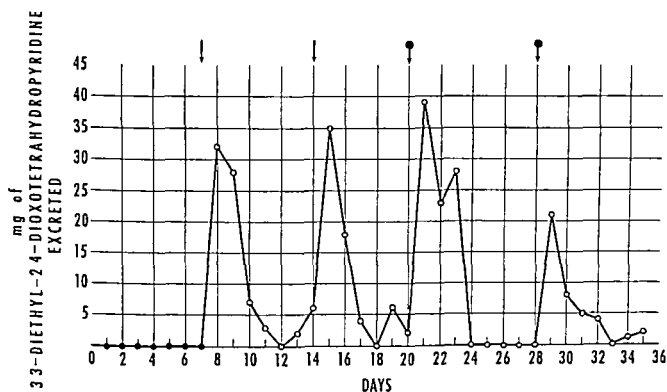


Fig. 1—Urinary excretion of 3,3-diethyl-4-dioxotetrahydropyridine (NU 903) for Subject L. K. receiving at weekly intervals two single doses of 0.6 Gm of 3,3-diethyl-4-dioxotetrahydropyridine (indicated by arrows) and two single doses of 0.75 Gm of 3,3-diethyl-4-dioxopiperidine (Sedulon) (indicated by arrows with dots). The initial estimation was carried out seven days prior to the administration of the first dose of NU 903 and subsequent assays were performed daily for five weeks.

Urine specimens of persons who had not received either the pyridine or the piperidine compound were found to show some difference in intensity of total fluorescence and residual fluorescence after hydroxylamine treatment. This observation was the reason for testing seven premedication voidings of each of the four subjects. Thus, average correction factors for hydroxylamine reactive

substances exhibiting irrelevant fluorescence were obtained. Calculated in milligrams of NU-903 per day, these correction factors were

SUBJECT		
E	K	$3.9 \pm S.D. 4.3$
M	L	$6.8 \pm S.D. 3.3$
D	S	$-0.6 \pm S.D. 6.0$
E	S	$7.7 \pm S.D. 3.9$

It can be seen that the amounts of hydroxylamine-reactive substances excreted differed from subject to subject, in one subject (D S) the fluorescence of the hydroxylamine-treated specimens was even slightly greater than that of the untreated urine specimens. However, in the individual subjects the quantities of irrelevant interfering substances were sufficiently constant from day to day to permit the use of the correction factors. Consequently all findings were corrected accordingly.

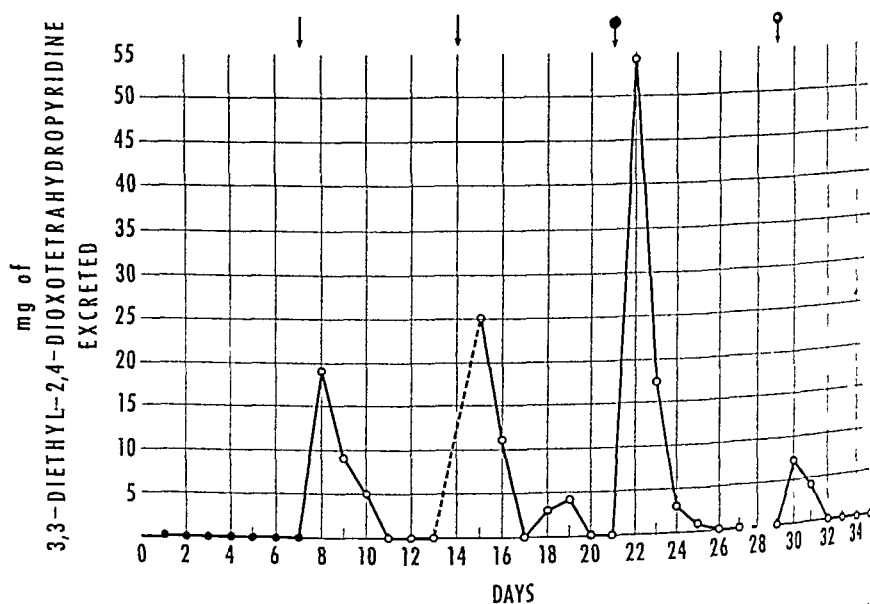


Fig. 2—Urinary excretion of 3,3-diethyl-2,4-dioxotetrahydropyridine (NU 903) for subject D S receiving at weekly intervals two single doses of 0.6 Gm of 3,3-diethyl-2,4-dioxotetrahydropyridine (indicated by arrows) and two single doses of 0.75 Gm of 3,3-diethyl-2,4-dioxopiperidine (Sedulon) (indicated by arrows with dots). The initial estimation was carried out seven days prior to the administration of the first dose of NU 903 and subsequent assays were performed daily for five weeks with the exception of two days (indicated by broken lines) when the specimens were lost.

Figs. 1 and 2 illustrate the procedure and the daily excretion values of NU-903 during the five weeks of experimentation for two subjects. The first two periods shown demonstrate the excretion of the pyridine compound following its administration, the last two periods show the elimination of the pyridine derivative following the administration of the piperidine compound. As can be seen, significant quantities of NU-903 were also eliminated following the ingestion of Sedulon.

The total amounts of NU 903 excreted in seven day observation periods by four subjects following each of two doses of NU 903 and two doses of Sedulon are recorded in Table III.

It can be seen from Figs. 1 and 2 that both drugs are excreted rapidly with the main portions of the eliminated amounts of NU 903 appearing in the first and second twenty four hour urine specimens collected after the intake of both compounds. Three to four days following the individual doses only negligible quantities of NU 903, if any, were detectable in the urine. Rapid elimination was observed also in the two other subjects.

TABLE III. URINARY EXCRETION OF NU 903 FOR FOUR NORMAL SUBJECTS AFTER SINGLE DOSES OF 0.6 GM. OF NU 903 AND AFTER SINGLE DOSES OF 0.75 CM. OF 3.3 DIETHYL 2,4 DIOXYPYPERIDINYL (SEDULON)

SUBJECT	E	E <sub>2</sub>	AVERAGE	E <sub>2</sub>	E	AVERAGE
E. K.	77	65	71	90	41	66
M. L.	66	41	54	84	15	50
D. S.	33	43	38	75	11	43
E. S.	60	51	56	43	40	42
Average			55			50

D. The total excretion of NU 903 in milligrams following the first dose of NU 903

L. The total excretion of NU 903 in milligram following the second dose of NU 903

S. The total excretion of NU 903 in milligrams following the first dose of Sedulon

E. The total excretion of NU 903 in milligrams following the second dose of Sedulon

As appears from Table III the total excretion values after each of the eight individual doses of NU 903 varied considerably ranging from 33 to 77 milligrams. However in a given subject the total excretion values recorded following the two individual doses of the pyridine compound were in fair agreement. For instance for Subject D. S. these values were 33 and 43 mg. respectively. The greatest difference was observed in subject M. L. who following the second dose of NU 903 excreted 38 per cent less than after the first dose.

It can be seen likewise from Table III that the total excretion values of NU 903 following each of the eight individual doses of Sedulon ranged from 11 to 90 milligrams. Thus these total excretion values varied even more than those recorded after the intake of NU 903. Moreover the total amounts of the pyridine compound eliminated by a given subject following the two individual doses of Sedulon differed appreciably. For example Subject D. S. whose excretion values after the two doses of NU 903 were in fair agreement excreted 11 mg. of NU 903 following the second dose of Sedulon as compared with 75 mg. after the first dose a reduction of 85 per cent.

In general the average of the two excretion values recorded for a given person following the administration of NU 903 was somewhat higher than the average of the two excretion values for that subject after the intake of Sedulon. The mean value of the average amounts of NU 903 excreted following 0.6 Gm. of NU 903 was 55 mg. or 9.2 per cent of the ingested dose and the mean value of the average amounts of NU 903 eliminated after 0.75 Cm. of Sedulon was 50 mg. or 6.7 per cent. It seems that the elimination of NU 903 following the intake of this drug is slightly greater than after the ingestion of the piperidine compound.

The urinary excretion of NU-903 following administration of this compound was studied in man by Polatin and co-workers<sup>6</sup>. These investigators, too, reported that following a single dose of 0.6 Gm the excretion level dropped promptly. For the six psychiatric patients whom they studied, the mean value of the total amounts eliminated over four days was 61 mg or 10.1 per cent of the ingested amount. This is in good agreement with the present findings.

Similarly, the extent of elimination of the pyridine compound reported by Kiautwald and associates<sup>5</sup> in dogs after the administration of 3,3-diethyl-2,4-dioxopiperidine approximates closely that determined for the four subjects in the present investigation, the excretion was 7 to 8 per cent of the amount fed to the animals as compared with a mean value of 6.7 per cent of the dose given to the human subjects.

Since the average excretion of NU-903 following the intake of this drug on the one hand and after the ingestion of Sedulon on the other appears to be of the same order of magnitude, it may be tentatively concluded that most or all of the piperidine compound administered is converted into the pyridine derivative.

The oxidation of the piperidine compound in the organism is suggestive of its mode of action. The belief that transformation of 3,3-diethyl-2,4-dioxopiperidine to the corresponding pyridine compound precedes its action is supported by both the somewhat slower onset and the longer duration of the effects of the former.<sup>3</sup>

#### SUMMARY OF PARTS I AND II

A new sedative drug, 3,3-diethyl-2,4-dioxopiperidine (Sedulon), was evaluated clinically in a total of sixty-two patients requiring sedation, twelve of whom had hypertension. Eleven of these subjects received 0.125 Gm three times a day as the initial dose, but only one derived good sedation at this dosage level. With a dosage of 0.25 Gm three times daily, good sedation was obtained in nearly all of the sixty-two cases. The effect of prolonged medication on the blood picture was studied in the twelve hypertensive patients receiving total daily doses of 0.375 and/or 0.75 Gm for two months. Hemograms at weekly intervals showed no significant changes in either the red or the white blood picture. In eight of these patients, Sedulon was continued so that they received the drug for from six months to almost one year with no untoward effect on the blood picture. In none of the sixty-two cases was the administration of Sedulon attended by untoward effects. The drug failed to reduce the blood pressure in the twelve patients with hypertension.

Both Sedulon and its dehydrogenation product, 3,3-diethyl-2,4-dioxotetrahydropyridine (NU-903), are rapidly excreted in the urine as NU-903. Four human subjects excreted an average of 9.2 per cent of each of two doses of 0.6 Gm of NU-903 taken seven days apart. Subsequently, they excreted average amounts of NU-903 equivalent to 6.7 per cent of each of two doses of 0.75 Gm of Sedulon taken at weekly intervals.



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## VARIATIONS IN DERMAL ABSORPTION WITH AGE

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THE activity of various bodily processes differs with age. It should be anticipated, therefore, that the rate or pattern of absorption of fluids from the skin will reflect these variations.

It is the purpose of this report to describe definite variations in the absorption of a dye from an intradermal site of injection, the variation apparently being related chiefly to the age of the subject rather than to specific disease entities.

A review of the literature reveals that various aspects of this problem have received considerable attention. McClure and Aldrich<sup>1, 2</sup> utilized the disappearance time of intradermally injected normal saline solution as a test of edema in nephritic children. They observed a more rapid disappearance (to palpation) of the wheal than in normal controls. This test was extended to a variety of pathologic conditions. A shorter disappearance time was observed in scarlet fever and diphtheria,<sup>3, 4</sup> lobar pneumonia,<sup>5</sup> jaundice,<sup>6</sup> tuberculosis,<sup>7</sup> serum sickness,<sup>8</sup> toxemia of pregnancy,<sup>9</sup> and peripheral circulatory deficiency.<sup>10, 13</sup> The disappearance time was found to be shorter in thyrotoxicosis<sup>14</sup> and prolonged in myxedema.<sup>15</sup> The work of Aldrich and McClure on children was confirmed by Feldman and Reitsneider<sup>16</sup> in adult nephritic patients. Berg<sup>1</sup> noted faster rate of disappearance in diabetic subjects, paralleling the severity of the diabetes, but this could not be confirmed by Levy and Rynes.<sup>15</sup> Olmstead<sup>19</sup> in a study of children with heart disease, found the test of prognostic value in severely ill or toxic patients.

Thompson<sup>12</sup> stated that the circulation had little to do with dermal absorption of saline solution and concluded that the actual mechanism was unknown. Olmstead<sup>19</sup> also advanced this view, noting that disappearance time post mortem was not increased. White and Irvine-Jones<sup>20</sup> likewise concluded that circulation played no part, on the basis of their observations of the effect of altered blood flow, temperature changes, and adrenalin. They did not agree with Olmstead, however, in regard to an unaltered disappearance time post mortem.

White and Irvine-Jones<sup>20</sup> and Taylor<sup>21</sup> found intradermally injected fluid in the corium. McMaster and Hudack<sup>22, 24</sup> who studied intradermally injected vital dyes in animals and humans, stated that intradermal injections are largely intralymphatic, the dyes entering lymphatics injured by the injecting needle. They observed that the dyes gradually spread through the superficial lymphatics.

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\*Our attention was drawn to this problem by observations of Dr. H. Sorter from the hospital that blood penicillin levels (following intramuscular administration) frequently were lower initially and higher late in old patients than in young patients.

and stated that differences in the spread were due to differences in skin texture in different subjects which caused the needle either to fail to reach a lymphatic plexus or to penetrate beyond it. In either case however the dyes reach large deeper lymphatics draining the injected area. Factors increasing lymph flow increased the rate of dye diffusion. Hudack and McMaster<sup>2\*</sup> pointed out that any local injection soon becomes a general one since the injected material enters the blood stream rapidly. This is borne out by the appearance of phenol red in the urine within fifteen minutes after intradermal injection.<sup>2</sup> White and Irvine Jones<sup>9</sup> were able to dismiss certain factors such as hydrogen ion concentration, potassium/calcium balance and osmotic pressure as being of significance in the absorption of normal saline solution from the skin. They felt that the disappearance of the intradermal wheal was due simply to diffusion and although they observed that artificially produced edema differed from nephritic or cardiac edema in that it did not produce an increased disappearance rate they were unable to find the effective absorptive factor. In this connection it is interesting that McMaster<sup>2</sup> found lymph stasis in cardiac edema and increased lymph flow in nephritic edema although in both instances the lymphatics are widened. In a recent article Kurziok and co-workers<sup>2</sup> referred to the work of Hoffman and Duran Reynolds on a factor which increased intradermal spread of various substances. Originally found in testicular extract it was also found in certain bacteria, snake venom and malignant tissue. This spreading factor acting on connective tissue by hydrolysis of hyaluronic acid salts is an enzyme hyaluronidase. These authors concluded that the factor (enzyme or enzymes) causing diffusion in the dermis is identical with that exhibiting hyaluronidase activity.

#### SUBJECTS AND METHODS

The subjects were not selected except that due to the nature of the test most were of the white race. Sex distribution was approximately equal. No severely ill patient was used and most were well enough to be either ambulatory or semiambulatory. One hundred and one of the subjects could be considered normal in regard to cardiovascular system and skin. This group included a number of subjects who were either in normal health (hospital staff) or could be assumed to be healthy such as surgical patients hospitalized for elective operative procedures. The conditions of the rest of the subjects (26 per cent) were such as would be encountered in the medical and surgical wards of any large general hospital. The experimental data obtained on these patients fell within the range of the normal controls in each group and it was apparent upon analysis of all data that age rather than a particular disease was the chief determining factor in dermal absorption. On the basis of the accumulated data it was seen that the entire series could be divided into groups each covering a period of fifteen years. The data on each age group were compared for statistically significant differences by the chi square test (Fisher) using a 5 per cent level of significance. Under Results we refer to this analysis when differences are called statistically significant.

One tenth cubic centimeter of a sterile solution of Evans blue in normal saline (0.05 mg per cubic centimeter) was injected intracutaneously on the middle third of the volar surface of the forearm using a short No. 25 hypodermic needle and a tuberculin syringe. One of us (J. V.) made all injections and readings. Four periodic measurements were made on each subject. This was done by placing a strip of clear celluloid film over the wheal or by staining and marking out the pattern. It was usually necessary to emphasize the dye outlines with

\*We are obliged to Dr. H. Silverstein for help in the statistical work.



TABLE II INITIAL WHEEL AREA

AGE GROUP (YR.)	PER CENT OF CASES WITH WHEEL AREA GREATER THAN 0.15 SQ. CM.
0-15	8
16-30	30
31-45	50
46-60	56
61-75	58
76-90	67

It is seen in Tables III and IV that the same general pattern continues at the end of four hours except in the 16 to 30 year age group where there is a trend toward a decrease in the wheal size which however, was found to have borderline statistical significance only.

TABLE III DISTRIBUTION OF WHEEL AREAS FOUR HOURS AFTER INJECTION

AGE GROUP (YR)	AREA OF WHEEL IN SQUARE CENTIMETERS																	NUMBER OF CASES IN EACH AGE GROUP																
	0	3	0	4	0	5	0	6	0	7	0	8	0	9	1	0	1		1	2	1	3	1	4	1	5	1	6	1	7	1	8	1	9
	NUMBER OF CASES																																	
0 15																																		13
16 30	1	4	9	2	6	2	1											1	1	1											28			
31 45				3	4	2	1	2											1	2	1											1	17	
46 60				2	5	5	4	1	6	1	2				1	2	3											32						
61 75				1	2	8	3	6	4	6	1	2	1	2	1	1	2											1	38					
76 90							2				1	2											1											6

TABLE IV WHEEL AREA FOUR HOURS AFTER INJECTION

AGE GROUP (YR.)	PER CENT OF CASES WITH WHEEL AREA GREATER THAN 0.75 SQ. CM.
0-15	39
16-30	20
31-45	44
46-60	50
61-75	63
76-90	67

It is further apparent, as shown in Table V, that visible dye remains in the injected area of a significantly larger number of persons in the younger age groups. The two groups from 10 to 14 and from 15 to 29 years of age had visible dye left in 90 to 97 per cent of the cases while the two older groups had visible dye in 57 to 79 per cent.

TABLE V NUMBER AND PER CENT OF CASES WITH DYE SEEN AT TWENTY FOUR AND FORTY EIGHT HOUR READINGS

	AGE GROUP (YR.)			
	10-14	15-29	30-39	60 AND OVER
Number of cases in group	14	29	48	47
24 Hour reading (%)	93	97	71	79
48 Hour reading (%)	93	90	57	53

## DISCUSSION

In young persons the initial wheal was smaller than in older persons (Tables I to IV). On the other hand, the dye remained longer in the skin of the younger subjects (Table V). Usually the size of the initial wheal increased in younger persons before it disappeared, while in older subjects it usually decreased steadily. These results are not readily explained. Lymphatic absorption may play a larger role than absorption into the venous blood.<sup>24</sup> It has been stated that the number of skin capillaries diminishes with age,<sup>2</sup> and one might assume that the number of lymphatic vessels diminishes likewise. Thus, in younger subjects lymphatic absorption may be greater in the initial phase of skin absorption than in older subjects.

Differences in texture of the skin between old and young persons are so distinct that they may well explain some of the phenomena observed. In the young, skin turgor is high and there are more elastic fibers present. In the old, turgor is low, elastic tissue is reduced, the epidermis and the deeper layers of the skin atrophy, and the skin has folds and consequently a greater surface area. In our tests it was easy to inject 0.1 c.c. of dye solution into the skin of older persons, but in the youngest group it was hard to push the needle through the skin and much more pressure had to be exerted to push the fluid into the skin. On a physical basis these differences may explain the larger initial wheals in the older subjects and the smaller ones in the younger subjects. Also, a possibly more rapid initial absorption in the younger group may be explained by the higher initial pressure in the wheal. On the other hand, the longer secondary persistence of dye in the skin of the younger group (Table V) may be explained by the relatively smaller area of the wheal and a consequently smaller absorbing area in which the dye is contained. In the older subjects the ready spread of the dye opens a larger absorbing area, initial absorption may be poor due to fewer lymphatic vessels, lesser pressure, possibly less tissue damage, less motility of the skin, and consequently less lymphatic suction, but the secondary stage of absorption (twenty-four and forty-eight hours) may be faster, due to the greater area occupied by the dye.

We have discussed mechanical factors mainly. Chemical factors, such as differences in hyaluronic acid salts and hyaluronidase, in histamine liberation, in water and salt content of the skin, in proteins that bind the dye, in macrophages, and so on, of old as compared with younger people are even more unknown than physical factors.

## SUMMARY

Differences in the absorption pattern of intradermally injected Evans blue in different age groups were demonstrated.

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## FURTHER STUDIES ON THE WELTMANN SERUM COAGULATION REACTION

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IN 1942 one of us (M. K.)<sup>1</sup> presented a study on the clinical significance of the Weltmann serum coagulation reaction, using as subject material the results of the reaction as tested in over 1,100 patients. Baker<sup>2</sup> shortly thereafter reported further results from Kraemer's laboratory. The present report is a continuation of those studies, covering the Weltmann reaction in over 3,000 cases.

Weltmann,<sup>3, 4</sup> in 1930, described a serum coagulation reaction which has shown itself to be of considerable clinical significance. He found that when normal human blood serum was diluted with distilled water 1:50 and boiled, the proteins failed to coagulate. However, when a small amount of electrolyte such as calcium chloride, magnesium chloride, or barium chloride was added, coagulation occurred. In certain pathologic conditions Weltmann noted that the serum required greater than normal concentrations of electrolyte in order for protein coagulation to occur, while in other conditions less than the normal concentration of electrolyte was required for coagulation. If 0.1 ml. of normal serum was added to 5 ml. of 0.4 per cent calcium chloride solution and boiled, the protein coagulated. However, if the serum was added to 0.2 per cent calcium chloride, coagulation did not take place.

Exudative and inflammatory processes so altered the blood serum that coagulation of the protein occurred only in concentrations of calcium chloride higher than 0.4 per cent. Conversely, fibrosing processes so altered the serum that coagulation occurred in concentrations of calcium chloride lower than 0.4 per cent. From these findings Weltmann devised the relatively simple test described below.

Various theories were propounded to explain the mechanism of the Weltmann reaction. Alterations in the following factors, either singly or in combination, were suggested as the basis of the reaction: the pH of the calcium chloride solutions employed, the pH of the serum, the serum calcium concentration, the total blood globulin, the albumin-globulin ratio, and the blood fibrinogen.<sup>5</sup> Dees studied the reaction extensively<sup>6, 8</sup> and suggested that the serum lipids are an important factor in the coagulation reaction. However, Scheelis and Levy<sup>5</sup> showed that the coagulation reaction is closely related to changes in the percentage of alpha globulin in the blood.

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## TECHNIQUE

Ten test tubes are set up in a rack and are numbered 1 to 10 from left to right. From a stock solution of 1 per cent calcium chloride ( $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ ) measured amounts are pipetted into each tube starting with 5 ml in the first tube and decreasing by 0.5 ml in each tube so that tube 10 contains 5 milliliters. Sufficient distilled water is added so that each tube will contain a total of 5 ml of solution, that is 4.5 ml of water are added to tube 10, 4 ml to tube 9 and so on in decreasing amounts to 5 ml in tube 2. This procedure results in ten dilutions of calcium chloride ranging from 1 per cent in tube 1 down to 0.1 per cent in tube 10.

To each tube is then added 0.1 ml of the blood serum to be tested. The serum must be unhemolyzed. After shaking the tubes in their rack are placed in a boiling water bath for exactly fifteen minutes. The rack of tubes is then removed from the bath and the number of tubes in which coagulation has occurred is recorded. The coagulum appears as a flocculation. Coagulation normally appears in the tubes of higher concentration (Tubes 1 to 6) and not in the tubes of lower concentration (Tubes 7 to 10). The number of the tube containing the most dilute solution of calcium chloride in which coagulation has occurred gives the reading for the test. Thus if coagulation occurs in tubes 1 through 6 the coagulation band is 6 (C B 6 or Weltmann 6). Care must be taken to determine the last tube in which *actual* coagulation has occurred since clouding or turbidity may occur in two or three tubes more dilute than the significant tube. With normal human serum coagulation usually occurs in the first 5 to 7 tubes (C B 5 or 6 or 7). When the coagulation band is less than 5 it is said to shift to the left and when it is more than 7 it is said to shift to the right.

In actual practice we have simplified this technique to ease the burden on the technician without sacrificing accuracy. If coagulation occurs in any tube by definition it will also occur in every tube to the left of it (that is in all higher concentrations). Therefore tubes 1, 2, 9 and 10 are not routinely set up since coagulation usually occurs in at least three tubes and rarely occurs in more than eight tubes. If, after the initial test is performed no coagulation has occurred in tube 3, tubes 1 and 2 are set up separately and the serum sample is tested in these tubes. Conversely if in the initial test coagulation has occurred in tube 8, tubes 9 and 10 are set up separately and the serum is tested in these tubes. For a single test this modification may seem of little value. However when an average of five samples of serum are tested simultaneously in parallel rows of tubes as is done in our laboratory the time and effort saved with this modification are considerable.

*Interpretation of the Test*—Weltmann<sup>3, 4</sup> demonstrated that in the presence of an exudative or inflammatory lesion there was a shift to the left of the coagulation band and that in the presence of fibrotic lesions there was a shift to the right. Subsequent investigators have corroborated these findings.<sup>6, 11</sup> However the bulk of significant results appears to be confined to the cases in which a shift to the left was obtained. This is in agreement with our findings.

Previous writers have found the coagulation band of normal sera to be rather fixed at 6 or 7. In our hands, however, we found we had to consider the normal range as varying from 5 to 7. Wachstein<sup>11</sup> also designated a coagulation band of less than 5 as being significantly shortened, regarding a coagulation band of 5 as being suggestively shortened.

#### CLINICAL STUDY

In Kramer's report, his results with the Weltmann reaction as performed on about 1100 patients were summarized. The present report includes the material plus the results obtained with sera from about 1870 additional patients, a total of 3954 Weltmann tests having been performed on about 3,000 consecutive private patients.

During the period covered in this report, a Weltmann coagulation test as well as a complete blood count, urinalysis, and sedimentation rate (Westergren or Cutler) was performed at least once on each patient in addition to other indicated diagnostic procedures. Most of the patients presented gastrointestinal problems and it is in this group that we have been able to follow the results of the Weltmann reaction with the greatest degree of accuracy.

Of the 3954 Weltmann tests performed, a total of 281 showed a coagulation band of 4 or less. It was possible to closely correlate the clinical or pathological findings with the low Weltmann reactions in 237 of these cases. In the remaining 44 cases no such correlation was possible. This latter group will be discussed later.

In Table I are listed the disease entities which were associated with a significant number of shortened coagulation bands.

TABLE I

DIAGNOSIS	TOTAL NUMBER OF CASES	PERCENTAGE OF LOW WELTMANN REACTIONS
Regional ileitis	9	55.6
Diverticulitis	16	50.0
Gastric carcinoma	29	41.4
Ulcerative colitis	51	35.2
Gastric ulcer	50	32.0
Choledocholithiasis	10	30.0
Carcinoma of the colon	11	27.3
Carcinoma of the rectum	18	16.7
Cholecystitis-cholelithiasis	296	10.8
Duodenal ulcer	371	10.4

It will be noted that in 55.6 per cent of cases of regional ileitis the Weltmann was 4 or less, this being the highest percentage in our series. In 50 per cent of cases of diverticulitis coli and in 41.4 per cent of cases of proved gastric carcinoma, the Weltmann was 4 or less. Cases of ulcerative colitis, gastric ulcer, and choledocholithiasis also showed significant percentages of low Weltmanns, 35.2, 32, and 30 per cent, respectively. Conversely, cases of duodenal ulcer, cholecystitis, and cholelithiasis showed the lowest percentages of low Weltmanns, although it has been our experience that in cases of penetrating duodenal ulcer and in ulcer with obstruction the incidence of low Weltmann reactions has been

relatively high. These results appear to confirm the finding that the coagulation band is shortened in the presence of exudative or inflammatory lesions. Ulcerative colitis is characterized by marked exudative and ulcerative inflammation. Gastric ulcer, penetrating duodenal ulcer and obstructive duodenal ulcers are generally associated with considerable inflammatory reactions and cholelithiasis is in many instances the cause of chronic low grade inflammation in the biliary tree. With regard to ulcerative colitis one might take the view that an incidence of 35.2 per cent positive Weltmanns is relatively low considering the characteristic pathology of the disease. It should be mentioned therefore that a number of the cases studied were quiescent at the time of study and have remained so.

Gastrointestinal malignancy, especially gastric malignancy is often associated with secondary ulceration and inflammation and the latter is probably responsible for the low Weltmann rather than the malignancy per se. We have observed a number of cases which would seem to substantiate this fact. Two of the cases will be cited briefly.

CASE 5919—The patient complained of increasing constipation of one year's duration. Proctoscopy (June 6, 1946) revealed a small fungating growth six inches above the anus. The Weltmann (June 10, 1946) was 4. A barium enema (June 10, 1946) revealed a filling defect of the rectosigmoid, a chest plate made the same day showed metastatic infiltration of the right lower lobe. The primary growth was removed by abdominoperineal resection. Three months later although the pulmonary metastases had increased in size and number the Weltmann (Sept. 19, 1946) was 6. These metastases were discrete and were associated with no inflammation or exudation, a low Weltmann was not expected once the ulcerating primary lesion was removed. On Dec. 24, 1947 the patient complained of pain in the left side of the chest on inspiration. A chest plate taken that day revealed a small pleural effusion. The Weltmann performed the same day was 3.

CASE 5660—Four months after partial gastric resection for gastric carcinoma and six months after radical mastectomy for primary carcinoma of the breast the Weltmann was 5 (July 27, 1946). Four months later (Nov. 16, 1946) the Weltmann was still 5. The patient was vomiting intermittently. In January, 1947 the patient developed signs of pleural effusion which proved to be metastatic in origin. The Weltmann (Jan. 16, 1947) was 1, an expected finding because of the appearance of the exudative lesion.

#### DISCUSSION

The Weltmann serum coagulation reaction appears to be of sufficient value as a laboratory diagnostic aid to warrant its routine use in gastroenterologic diagnosis. Admittedly it is a nonspecific reaction but this fact does not detract from its utility. In the presence of exudative or chronic inflammatory reaction the Weltmann coagulation band is shifted to the left in a significant number of instances. It has been our experience and that of others<sup>2</sup> that in general, when even there is a low Weltmann the sedimentation rate is elevated but there is no correlation between these findings. With a Weltmann of 4 one may find a sedimentation rate of 20 or 85 while in the same sedimentation rate may be noted with a Weltmann of 6 or 2. The sedimentation rate is often influenced by a number of factors many of them of relatively minor nature, and it thereby loses diagnostic significance. The Weltmann reaction however when positive

that is when 4 or less, usually indicates, in gastrointestinal complaints, an inflammatory lesion of serious import and rapidly reverts to normal when this lesion is healed or removed. It is on this account helpful in both diagnosis and prognosis. The following cases will illustrate this point.

CASE 6898—On Oct. 6, 1947, the Weltmann was 4 and the sedimentation rate, 4. Posterior wall gastric ulcer and duodenal ulcer were found on x-ray examination. Gastroscopy revealed a benign lesion. On Nov. 18, 1947, after six weeks of treatment, the patient was asymptomatic with a Weltmann of 6 and a sedimentation rate of 16. The nodule was still present but much smaller.

CASE 6512—April 1, 1947, there was a history of recurrent epigastric pain of four years' duration which was relieved by food. The Weltmann was 4 and the sedimentation rate, 22. Gastrointestinal x-ray films showed no evidence of lesion but the patient was placed on an ulcer type of therapy. On May 20, 1947, there were no complaints, there was a weight gain of five pounds, the Weltmann was 7, the sedimentation rate 11. On June 17, 1947, there was occasional slight epigastric discomfort, the Weltmann was 4 and the sedimentation rate, 18. On Sept. 18, 1947, a posterior wall gastric ulcer was found on repeat x-ray study (confirmed at operation).

In 44 cases with positive Weltmanns we were unable to determine the cause of the positive reaction. Almost without exception this group consisted of patients who were seen only once and therefore an adequate diagnostic work-up and follow-up were not possible. More thorough study probably would have revealed the pathologic basis for the positive test in all or most of these patients but the possibility of false positive reactions in this group cannot be ruled out. However, in the 237 patients in whom adequate study was performed, a small number of positive tests was obtained in inflammatory states such as acute severe gingivitis, acute tonsillitis or acute sinusitis, with subsidence of the inflammation the Weltmann reactions in these instances rapidly reverted to normal. The incidence of actual false positive reactions, that is positive reaction in the absence of any demonstrable inflammatory or exudative lesion must be very slight.

It would be unwise to attribute to the Weltmann reaction values which it does not have. Many cases of serious disease of the gastrointestinal tract have been diagnosed in the presence of a normal Weltmann. However, when the Weltmann has been positive, complete diagnostic work-up has usually revealed a lesion of importance. This has led us to the clinical assumption that a patient with a low Weltmann reaction has a serious illness until absolutely proved otherwise. In a number of instances the cause of a low Weltmann has been traced to important lesions outside the gastrointestinal tract after gastrointestinal work-up had revealed a lesion sufficient to explain the presenting symptoms but not sufficient to explain the low Weltmann reactions.

#### SUMMARY AND CONCLUSION

The Weltmann serum coagulation test and a modification for its simplification in routine use are described.

The results of the Weltmann test as performed in over 3,000 consecutive patients and the significance of the reactions have been analyzed.

The test appears to be of considerable value in indicating the presence of exudative or inflammatory disease of the gastrointestinal tract including neoplastic lesions when the latter are accompanied by ulceration or exudation

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# ELECTROPHORETIC STUDY OF THE BLOOD SERUM FROM LYMPHOGRANULOMATOUS PATIENTS

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THE present investigation was undertaken in order to determine whether electrophoretic analysis of blood sera withdrawn from patients with Hodgkin's disease would demonstrate patterns peculiar to this disease. Such findings might be useful in diagnosis and prognosis and might throw light upon the nature of the illness. Impetus was given to our studies by recent electrophoretic investigations of human sera obtained from normal and diseased subjects indicating that the protein fraction reflected the state of the organism as a whole and that in one instance (in nephrosis) a strikingly characteristic curve was obtained.

## EXPERIMENTAL

*Materials and Methods*—Blood samples were withdrawn under sterile conditions from patients under observation and treatment at the Hodgkin Disease Research Clinic of St. Vincent's Hospital. These samples were allowed to clot and were stored in the refrigerator until examination\*. The diagnosis of Hodgkin's disease was in every instance proved by biopsy and in some instances further confirmed by necropsy.

For electrophoretic examination 5 to 10 ml. of blood serum were diluted with two volumes of sodium barbiturate-barbituric acid buffer of an ionic strength of 0.1 and a pH of about 8.5. The diluted serum was then dialyzed in cellophane tubing against 2 liters of the same buffer for two to three days in the refrigerator. Before placing the dialyzed serum in the electrophoretic apparatus, it was centrifuged in an angle centrifuge to sediment any suspended particles; the tall section analytic cell of Tschus Longworth<sup>2</sup> of 11 ml. protein solution capacity was employed throughout. The apparatus was a commercial instrument closely based upon a design by Longworth<sup>4</sup>. The electrophoretic diagrams were recorded photographically with the Schlieren scanning method<sup>4</sup>. Visual observations in the course of the experiments were made with the Svenson-Philpot cylinder lens angular diaphragm technique<sup>3</sup>. The temperature of the thermostat was 13° C., the voltage gradient about 6.6 v. per centimeter, and the time of electrophoresis approximately 14,000 seconds.

As light source, a mercury high-pressure burner (G. E. H-4) was used in the case of clear and little-colored serum specimens, and a single filament tungsten source (G. E. 35 A/14 T, 10 V) for the examination of colored or opalescent sera.

The hydrogen ion concentration and the conductivity of the solutions were measured with a glass electrode pH meter and an electronic conductivity bridge respectively.

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\*The electrophoretic experiments were performed in the Institute of Polymer Research, Department of Chemistry, Polytechnic Institute of Brooklyn.

For quantitative evaluation the original photographic negatives were enlarged about three times by projection and the enlarged patterns were traced by hand. The tracings were then measured with a polar planimeter after the areas had been divided by a vertical line through the minima and subdivisions assigned to the individual components.<sup>1</sup> Relative concentrations in per cent were computed in terms of the ratio of these areas to that of the total area under the curve after the stationary, monomalous boundaries had been eliminated from the diagram. In the majority of cases the calculations are based on the pattern recorded from the descending boundaries but in two instances the ascending boundary patterns were also evaluated (see Tables I, II and III). It is realized that the procedure here adopted disregards the small differences in the refractive increment of the various proteins of blood serum and also the contribution of protein to the boundary anomalies. It is felt however, that in comparison with the other possible sources of error in electrophoretic determinations these factors are of only secondary importance.

The electrophoretic mobilities of the individual components appeared to be of the same order as those of normal serum hence no systematic measurements of mobilities were undertaken.

In order to avoid any bias on the part of the investigators the serum samples were submitted for examination without any clinical information. In some instances several specimens from the same patient obtained after varying intervals of time, were studied in the electrophoretic apparatus.

The sera specimens examined were obtained from twenty seven patients suffering from Hodgkin's disease eighteen men and nine women. Duration of affliction and severity of manifestations were not alike in any two cases. The age range was from 13 to 61 years and the duration range from 1 to 5 years. There were further variations, namely in the rapidity of development of disease in the state of nutrition of the patients, and so forth.

*Observations and Results*—A total of thirty three serum samples was examined electrophoretically under strictly comparable experimental conditions. The electrophoretic patterns obtained fell into three groups.

The first group (A) yielded diagrams somewhat similar to those of normal individuals. The albumin globulin ratio was greater than unity and the absolute as well as the relative concentrations of the individual protein components, excepting the  $\alpha$  globulin fraction were found to be near the limits of variation found previously for normal human serum both in this laboratory and by other investigators. The electrophoretic data pertaining to this group are compiled in Table I. For the purposes of comparison the mean values as determined by Dole and Brun on the blood plasma of fifteen normal young adult male subjects under similar experimental conditions are included in Table I. The fibrinogen values are omitted because serum was used in the present study. This component amounts to approximately 5 per cent of the total area of the plasma protein pattern.

It will be noted that the values for the different serum components vary appreciably from case to case as does also the albumin globulin ratio. On the

TABLE I ELECTROPHORETIC COMPOSITION OF SERA FROM LAMINOGRAPHIC PATIENTS IN GROUP A, THE VALUES HERE RECORDED ARE SIMILAR TO THOSE FOR NORMAL AND NEARLY NORMAL BLOOD SERUM

PATIENT	AGE (yr.)	SEX	DURATION OF ILLNESS, ONSET TO EXPERIMENT	CLINICAL STATUS	A/G RATIO	RELATIVE CONCENTRATION IN PER CENT OF TOTAL AREA				
						ALBUMIN	ALPHA 1	ALPHA 2	BETA	GAMMA
421	32	M	11 mo	Good	1.1	56.7	4.73	12.5	11.3	14.8
422	21	M	5 yr.	Fair	1.7	62.4	6.11	11.8†	11.0	5.65
427	24	M	10 mo	Good	1.6	61.3	5.24	7.75	15.1	10.5
429 a	33	M	24 mo	Good	1.7	63.0	5.84	14.5	10.4	6.37
443 a	33	M	11 mo	Good	1.1	51.9	5.7	11.3	15.6	15.1
457	29	M	2 yr.	Fair	1.0	50.0	7.0	15.3	16.4	11.3
467 a	13	M	6 mo	Good	1.5	60.4	7.5	9.5	11.6	11.2
471	35	M	17 mo	Good	1.2	55.1	1.6	10.2	12.5	17.8
467 b	13	M	7 mo	Good	1.8	64.0	5.0	14.1	7.5	9.3
480 a	19	M	9 mo	Good	1.4	58.3	6.9	13.7	7.9	13.3
483	29	M	25 mo	Good	1.2	50.6	7.5	14.3	16.9	10.7
443 b	33	M	11 mo	Good	1.4	62.0	5.7	11.3	5.6	15.4
474	27	M	12 mo	Good	1.3	57.2	7.5	10.9	13.4	11.1
Mean value					1.4	57.9	6.1	12.3	11.9	11.8
Average deviation					±.2	±1.0	±.9	±2.0	±2.8	±2.8
Normal values for plasma (Dole and Bruno)†					1.53	60.3	4.6	7.2	12.1	11.0

\*Relative concentration in per cent of total area as calculated from diagrams obtained in the descending limb of the cell

†Component split in two peaks

‡Phase values represent the average for the ascending and descending limbs of the cell. For serum these figures should be increased by about 5 per cent



whole, the albumin globulin ratios of the sera grouped together in Table I are somewhat lower and the relative concentrations of the  $\alpha_2$  and of the  $\gamma$  globulin fractions are higher than the values reported by Dole and Brun for normal serum. This is true even if allowance is made for the absence of fibrinogen in the samples studied by us. How significant these deviations are, namely how far they may be due to the fact that these sera were all obtained from patients with Hodgkin's disease, cannot be stated at this time. The same holds true for the differences in quantitative composition of the specimen examined as experiment 467 a and that withdrawn from the same patient four weeks later and examined as Experiment 467 b. A pair of electrophoretic diagrams representative of this group of sera is reproduced in Fig. 1.

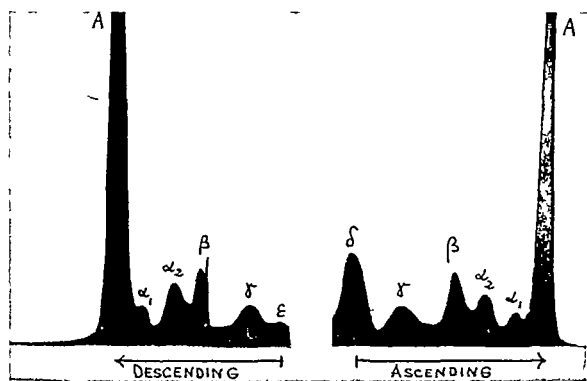


Fig. 1—Electrophoretic diagram of serum from a patient with Hodgkin's disease falling into the A group (Experiment 471 Table I) normal or nearly normal pattern as to general outline absolute and relative concentration of components and electrophoretic mobility

The second group (B) of sera, listed in Table II shows the following features in common. The  $\alpha$  globulin fraction is present in an amount which is significantly higher than in normal blood serum. The increase is limited either to the  $\alpha_1$  or the  $\alpha_2$  globulin component or it involves both of these protein fractions of high electrophoretic mobility.

With a single exception the albumin globulin ratio is below unity. In some instances the  $\beta$  globulin component is also increased in relative concentration (Experiments 381 382 419).

The significance of the increase in the  $\alpha$  globulin components here observed will be discussed below in the light of the clinical findings.

Diagrams typical for this second group of sera are shown in Fig. 2.

In the third group (C) of sera examined and compiled in Table III the common denominator is a significant increase in the relative concentration of the  $\gamma$  globulin fraction. The albumin globulin ratio is below unity with one exception Experiment 459.

TABLE II ELECTROPHORETIC COMPOSITION OF SERA FROM LYMPHOGRAULOMATOUS PATIENTS IN GROUP B, IN THIS GROUP THE RELATIVE CONCENTRATION OF EITHER ONE OR BOTH ALPHA GLOBULIN COMPONENTS IS INCREASED AND THE A/G RATIO IS BELOW UNITY

EXPERIMENT	AGE (YR.)	SEX	DURATION OF ILLNESS, ONSET TO EXPERIMENT	NUTRITION AND CLINICAL STATUS	A/G RATIO	RELATIVE CONCENTRATION IN PER CENT OF TOTAL AREA			
						ALBUMIN	ALPHA 1	ALPHA 2	GAMMA
373	37	M	17 mo	Poor, Terminal	0.91	47.7	4.7	14.8	16.2
378	47	M	14 mo	Poor, Terminal	0.73	42.1	10.4	13.7	15.3
381*	39	F	5 mo	Poor, Terminal	0.46	31.6	15.4	19.0	18.5
382*	38	F	19 mo	Poor, Terminal	0.67	39.5	11.7	13.4	20.5
382*	38	F	19 mo	Poor, Terminal	0.66	40.0	12.7	12.9	22.3
419	27	M	21 mo	Poor, Terminal	0.52	35.0	18.1	17.0	22.0
420	22	M	30 mo	Poor, Terminal	0.55	35.4	14.0	17.9	20.2
430	56	F	21 mo	Poor, Terminal	0.55	35.7	18.3	21.5	12.8
438	43	F	5 mo	Poor, Terminal	0.49	32.7	13.4	20.3	11.4
463	61	F	6 wk	Poor, Terminal	0.36	26.5	15.7	23.0	14.6
466	24	M	21 mo	Poor, Terminal	0.54	35.2	20.6	19.3	17.0
Mean value					0.59	36.5	14.1	17.5	10.3
Average deviation					±16	±4.2	±3.3	±2.9	±3.5
									±2.7

\*Areas calculated from ascending boundaries

TABLE III ELECTROPHORETIC COMPOSITION OF SERA FROM LYMPHOCYTOBLASTIC PATIENTS IN GROUP C IN THIS GROUP THE RELATIVE CONCENTRATION OF GAMMA GLOBULIN IS INCREASED WHILE THE A/G RATIO IS, AS A RULE BELOW UNITS

EXPERIMENT	AGE (yr)	SEX	DURATION OF ILLNESS, ONSET TO EXAMINATION	NUTRITION AND CLINICAL STATUS	A/G RATIO	RELATIVE CONCENTRATION IN PER CENT OF TOTAL AREA				
						ALBUMIN	GLOBULINS			GAMMA
							ALPHA 1	ALPHA 2	BETA	
308	4	M	2 yr	Fair	0.83	45.0	5.3	10.3	1.2	26.7
442	19	F	1 yr	Good	0.61	35.2	8.9	13.2	13.4	26.2
444	31	M	3 yr	Fair	0.67	40.4	6.8	13.0	14.5	25.4
420 b	33	M	2 mo	Good	1.07	56.0	3.6	9.2	14.0	16.7
420 c	33	M	28 mo	Good	0.92	48.8	8.9	1.7	10.2	19.5
400 a	40	F	1 yr	Good	0.88	36.6	10.4	13.7	11.1	28.2
400 b	26	F	13 mo	Good	0.68	40.3	10.3	13.6	10.7	20.1
401	20	F	4 yr	Good	0.88	46.4	8.8	13.8	11.3	19.4
462	43	F	3 mo	Good	0.57	36.3	9.6	16.3	14.9	22.9
480 b	19	M	9 mo	Good	0.71	41.6	9.5	14.0	13.1	21.4
Mean value					0.70	41.1	8.2	13.0	12.0	23.2
Average deviation					±14	±5.0	±1.5	±1.4	±1.4	±1.2

\*Gamma globulin plus  $\alpha$  component.

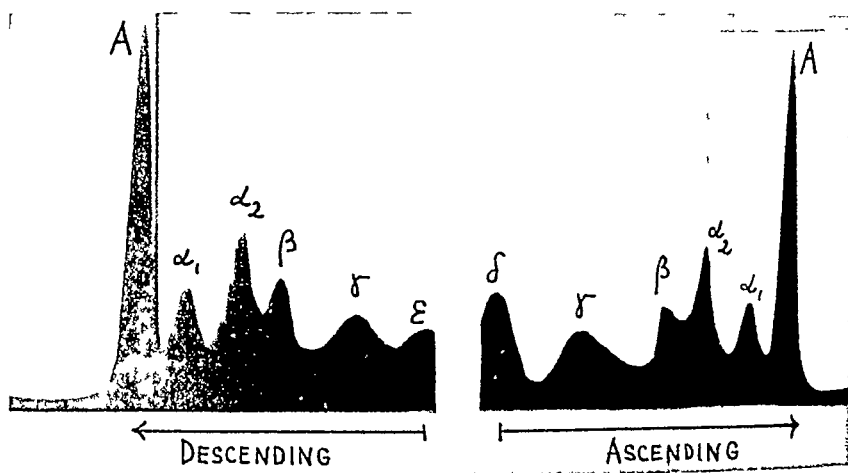


Fig 2—Electrophoretic diagram of serum from a patient with Hodgkin's disease falling into the E group (Experiment 438 Table II) the pattern is characterized by relative decrease in albumin and corresponding increase in total globulin concentration (inverted albumin globulin ratio) as well as by strikingly enlarged alpha-1 and alpha-2 globulin areas

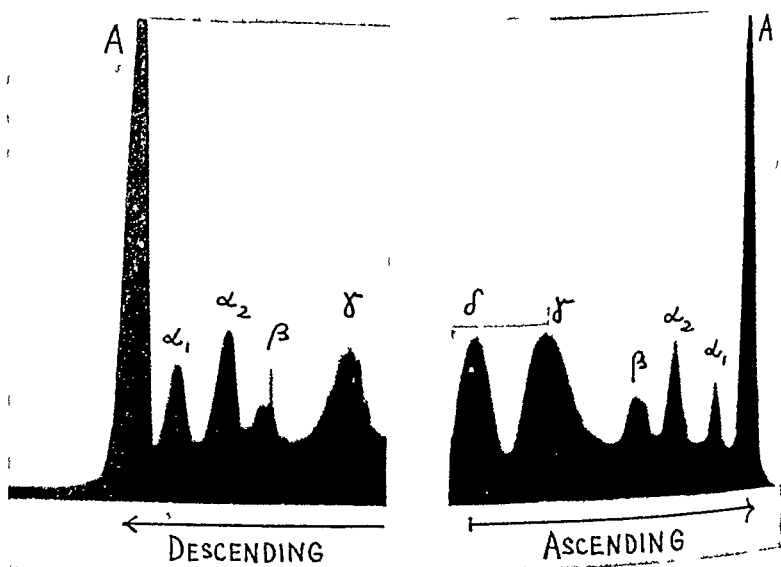


Fig 3—Electrophoretic diagram of serum from a patient with Hodgkin's disease, falling into the C group (Experiment 460-b Table III) the pattern shows abnormally enlarged gamma globulin area in addition to increase in the alpha globulin components

Experiments 429-b and 429-c were carried out on two blood samples from the same person, an interval of four weeks ensuing between collection of samples

Diagrams of the Group C type are reproduced in Fig 3

In one instance, Experiment 368, an extra globulin component of an electrophoretic mobility intermediate between that of gamma and beta globulin

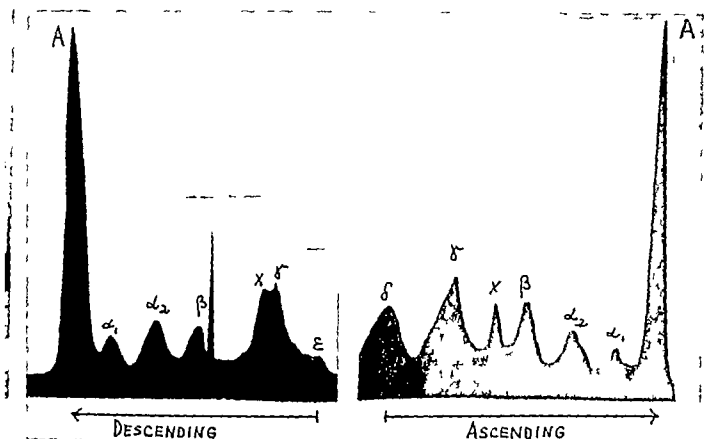


Fig 4—Electrophoretic diagram of serum from a patient with Hodgkin's disease falling into the C group (Experiment 365 Table III). In addition to the increase in gamma globulin area, characteristic for this group the pattern also shows the presence of an additional protein component, labeled  $x$  on the diagram with a mobility intermediate between beta and gamma globulin. The  $x$  and gamma globulin maxima are fully resolved only in the ascending limb of the cell.

was observed (see Fig 4). This component, labelled  $x$  in the diagram was fully resolved from the  $\gamma$  globulin boundary only in the ascending limb of the cell.

Comparison of the tables will show that the distinction between the three groups of sera is not too sharply defined in all instances. As would be expected sera of intermediate type were encountered in this study rendering their classification difficult and somewhat arbitrary. This indicates the existence of transitions from one group to another which, perhaps reflect transitory changes in the clinical state of the patient (see below). Thus the specimen examined in Experiment 429 b might also be placed in the A group while the specimen from the same patient used in Experiment 429 c clearly falls in the C group. Other instances are the sera studied in Experiments 460 a and 460 b which show increases in the relative concentrations of the alpha as well as of the gamma globulin fractions, and those analyzed in Experiments 373 and 457.

#### CLINICAL FINDINGS

*Group 1*—The thirteen sera in Table I were obtained from eleven patients. All were ambulatory, in a good nutritional state and free from such symptoms of Hodgkin's disease as noticeable enlargement of lymph nodes. Experiments 467 a and 467 b were performed on specimens taken from the same patient

at an interval of one month, while Experiments 443-a and 443 b were done on the serum of another patient with an intervening period of four months. Since in one patient (Experiment 467-a) the disease had been manifest for only six months and in another (Experiment 422) for five years, the duration of the disease does not seem, of itself, to be responsible for the shape of the electrophoretic pattern. All patients in Group A had at one time or another received x-ray therapy, two of the patients were under x-ray treatment at the time the serum was withdrawn from them. With the exception of one patient (Experiment 422), who died ten months after his serum was examined, all patients are alive at the present time. Four of them have not required further therapy in the intervening period, their red cell sedimentation rates are normal or only slightly increased above normal value. Five other patients (Experiments 429, 457, 461, 480 and 483) have since been treated with mustard gas following enlargement of the lymph nodes, recurrence of pain, and loss of weight. In all instances the response to the treatment has been satisfactory.

*Group B*—In striking contrast to the sera in Group A, the ten sera falling into Group B were obtained from patients in an advanced stage of Hodgkin's disease, terminal cases actually. The patients were very ill at the time the specimens were obtained. They were bedridden and emaciated and had fever. All the patients in this group died within three days to five months after the blood samples were withdrawn for electrophoretic study.

*Group C*—More than one electrophoretic experiment was performed on the serum of three of these patients. Experiments 460 a and 460 b were carried out on the serum of the same patient with an interval of one month, Experiments 480-a and 480-b on the serum of another patient with an interval of two months (Experiment 480-a will be found in Table I). In a third instance of multiple analysis the first experiment fell into Group A (see Experiment 429 a, Table I), while subsequent experiments on serum samples withdrawn from the same patient after three and four months (Experiments 429 b and 429 c, respectively) were classified as Group C patterns (see Table III). Actually the diagram obtained in Experiment 429-b represents a borderline case between Group A and C sera. In neither of these patients were we able to discover a clinical basis for these variations in the electrophoretic pattern.

All of the patients in this group were ambulatory and in good nutritional state. Considered as a group, however, they represented more severe states of Hodgkin's disease than those appearing in Group A, they suffered more from fatigue (particularly patients furnishing the serum specimens for Experiments 461, 462, and 460-b). The patient represented by the sample in Experiment 368 exhibited recurrent enlargement of lymph nodes so severe as to require the institution of x-ray therapy. Two of the patients in Group C have died (Experiments 318 and 462). One other patient (Experiment 442) has been bedridden nine months and is at the present time in a terminal stage of the disease.

Attempts to correlate the electrophoretic patterns recorded in this study with individual factors, such as duration of illness, x-ray therapy, anemia, age of the patient, or sex, failed. However there is an indication that the Group B pattern prevailed in those instances in which the disease ran a rapidly fatal course. Five of the sera in this group (B) were obtained from patients who had had Hodgkin's disease less than a year, while the remaining five had been ill for about one year. This is in contrast to the patients represented by Table I, five of whom had been ill for less than a year, three for a year, three for two years, and one for three to five years. Group C shows a more general distribution as to disease duration, since two each had been ill for less than one year, for one year, and for two years and one each for three and five years.

#### DISCUSSION

The observations made in this study indicate that the electrophoretic pattern of the serum proteins is nonspecific for Hodgkin's disease. Thus none of the thirty-three analyses made by us produced a pattern peculiar to Hodgkin's disease. Three types of curves—designated A, B, and C—were however obtained, and each curve could be more or less correlated with the clinical state of the patient. Thus the A curve was obtained from patients whose nutritional state was and is to date, normal. The B curve, characterized by elevated alpha globulin components, was produced by terminal stage patients, those who were cachectic, anemic, febrile and edematous. Finally, the C curve, characterized by elevated gamma globulins, was furnished by patients in a stage intermediate between the two others, not terminal but definitely more advanced than that of patients in Class A.

Comparison of our findings with those obtained from sera of patients with tuberculosis failed to show any parallelism between tuberculosis and Hodgkin's disease. In the former disease the alpha globulin rose early in the course of the illness, while in the latter disease a rise of the same fraction occurred terminally. In tuberculosis<sup>6</sup> the increase in gamma globulin appeared to be related to immunologic processes while in Hodgkin's disease no such conclusion could be drawn.

Of further interest is the recorded increase of alpha globulin in pneumonia<sup>7</sup> and in other febrile diseases which are accompanied by tissue breakdown.<sup>8</sup>

In the course of our studies the question arose as to whether blood sera patterns were reversible from the B and C types to A. Only one example was found, Experiment 480 b, which fell into the Group C. A repeat test on a blood sample withdrawn from this patient one month later resulted in an A curve. The maintenance of a perfect nutritional state in this patient sixteen months after the tests despite recurrence of massive lymph node enlargement would indicate a relationship between nutritional state and the electrophoretic curve rather than a relationship between lymph node enlargement and the electrophoretic serum pattern.

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## SPECIAL ANNOUNCEMENT

The following is inserted at the request of the Cutter Laboratories

Berkeley, Calif  
May 6, 1948

For Immediate Release

Superseding our previous release dated May 5, 1948 is the following statement from Dr R K Cutter, president, of Cutter Laboratories: "Contamination has been found in another and entirely different glucose solution, dextrose 10 per cent in Ringer's, according to an announcement made today by Dr R K Cutter, president, of Cutter Laboratories. The company is cooperating with the Food and Drug Administration, and is requesting the assistance of health departments throughout the country, in immediately recalling from hospitals Cutter's entire line of dextrose and other solutions for mass intravenous injection. Company officials believe that discovery of this new contamination makes questionable the use of any product produced in their intravenous solutions department until this entire contamination difficulty is solved. The other products produced in this department are concentrated dextrose, distilled water, sodium citrate, normal saline solutions in 50 and 100 cc bottles, as well as all flasks supplied by Cutter for community blood and plasma banks.

"The reason for this contamination is still unknown, and until they have the positive answer, Cutter feels this is the only step that can be taken in the interest of public safety. In the meantime, arrangements are being made to supply hospitals with solutions of other manufacturers."

Cutter Laboratories.



# THE TISSUE DISTRIBUTION OF RADIO ANTIMONY INHALED AS STIBINE

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BETHESDA, MD

## INTRODUCTION

RECENT problems in the field of tropical diseases have served to emphasize our present limitations in their treatment. To further the therapeutic use of compounds of antimony in these diseases a better knowledge of the physiologic and pharmacologic properties of the element is necessary. New and more precise methods of assay for antimony have been recently developed which can be used for studies *in vitro* and *in vivo*.<sup>1, 2</sup>

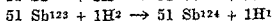
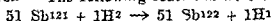
Since the toxicity of a drug is, in general, a function of both concentration and time of retention, a given agent is likely to show varying toxicities to different organ systems of the host and parasite depending upon the extent and chemical state of local accumulation and the rate at which it occurs.

Inhalation of a gaseous compound of antimony, stibine ( $SbH_3$ ) has been reported effective in reducing the parasite count in chicks infected with *Plasmodium gallinaceum*.<sup>3</sup> It therefore seemed of interest to learn the concentration of antimony as a function of time in the blood and tissues of chicks (both normal and infected with *P. gallinaceum*) and in guinea pigs after treatment with stibine containing radioantimony.

## METHODS

A radioactive tracer method was selected because it permitted rapid detection and accurate measurement of amounts as small as micrograms of the element per gram of tissue without the tedious chemical manipulations of microchemical procedures. Radioactive antimony is one of the more favorable elements for this purpose, since two relatively long half life isotopes can be produced efficiently.

The antimony isotopes were prepared by the bombardment of antimony as a probe target in a sixty inch cyclotron\*. The following reactions were utilized:



The two isotopes have a half life of 28 and 60 days, respectively and were used in the mixed form as produced.

Radioactive antimony was added to powdered magnesium in the ratio of 1:5 and fused in a furnace under  $N_2$  to produce an alloy from which stibine could be generated at will by dropping a known amount of the alloy into a 50 per cent hydrochloric acid (HCl) solution in a special container†. The chamber used for exposing animals to various concentrations of the gas was designed to permit rapid withdrawal of gas samples and of exposed animals during the experiment.

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The opinions or assertions contained in this article are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

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†We are deeply indebted to Dr. S. H. Webster of the National Institute of Health, Bethesda, Md. for furnishing us with the basic data for this method of producing stibine.

Animals were removed from the chamber at the desired intervals, exsanguinated, the various organs and tissues dissected and weighed immediately. Blood samples were at once centrifuged and the desired components taken up in 1 ml Luer syringes. Organs and tissues were ground with small glass mortars and pestles and the samples measured by volume in Luer syringes. Occasionally the quantity of tissue was insufficient, so that organs for several animals were pooled. The measuring cups were of Lucite and were kept free of radioactive contamination as shown by check with Geiger counters. Each cup was used only once during any given experiment. A standard volume (0.4 ml) of tissue or blood was used for measurement wherever possible. The radioactivity of the sample was measured by a beta ray counter at a standard distance. The conversion of counts per second to micrograms of antimony was made by reference to a standard sample containing a known amount of antimony, the radioactivity of which had been measured in a manner identical to that of the biologic samples.

#### PROCEDURES AND RESULTS

Four groups of from forty to seventy chickens (7 and 9 days old) and one set of twenty guinea pigs were gassed with stibine for a period of about fifty minutes, removed from the gassing chamber at the desired time, and sacrificed for analysis. Two groups of the chickens and the guinea pigs were from normal stock, a third group of chickens was found upon autopsy to have a respiratory infection, and the fourth had been heavily infected with *P. gallinaceum*. Parasite counts were made on each chicken in the fourth group prior to the gassing period. Stibine gas concentration for these experiments was about 25 parts per million.

Time-concentration curves were obtained for blood and certain of its fractions and for spleen, liver, kidney, heart, and brain. Each point in the curves is an average value for several individuals (two to six with the chicks, two with the guinea pigs). Some of the fluctuations observed are obviously due to sampling errors and the small number of animals used for each point. It is evident that the greatest variability occurs in muscle and, in general, the least in liver.

TABLE I. ANTIMONY CONCENTRATION IN NORMAL CHICKS TREATED WITH STIBINE GIVING THE INDIVIDUAL DETERMINATIONS FOR ONE SERIES AND THEIR AVERAGES

TIME	TISSUE	MICROGRAMS ANTIMONY PER GRAM WET TISSUE			AVERAGE MICROGRAMS ANTIMONY PER GRAM WET TISSUE	STANDARD DEVIATION	TISSUE	MICROGRAMS ANTIMONY PER GRAM WET TISSUE			AVERAGE MICROGRAMS ANTIMONY PER GRAM WET TISSUE	STANDARD DEVIATION
0 min	Heart	5.82	6.63	5.97	6.14	0.56	Muscle	1.0	1.9	1.3	1.4	0.51
15 min	Heart	6.81	8.95	7.40	7.72	1.41	Muscle	1.7	2.6	1.9	2.1	0.51
30 min	Heart	7.44	8.58	7.69	7.99	0.68	Muscle	2.4	2.6	1.9	2.3	0.41
1 hr	Heart	7.03	6.15	6.74	6.64	0.56	Muscle	2.0	2.3	1.2	1.8	0.34
2 hr	Heart	6.08	5.19	5.52	5.60	0.56	Muscle	1.6	1.6	2.1	1.7	0.21
4 hr	Heart	4.90	4.16	3.83	4.30	0.69	Muscle	1.3	1.1	1.5	1.3	-
8 hr	Heart	3.66	4.12	-	3.89	0.32	-	-	-	-	-	-
0 min	Liver	19.7	25.6	29.1	24.8	5.84	Lung	12.4	11.8	12.8	12.3	0.63
15 min	Liver	25.5	25.8	27.1	26.2	1.03	Lung	11.8	15.5	-	13.6	1.71
30 min	Liver	27.9	28.5	24.3	26.9	3.00	Lung	11.0	9.3	10.8	10.4	1.01
1 hr	Liver	29.1	30.1	27.2	28.8	1.83	Lung	12.1	9.2	-	10.7	1.31
2 hr	Liver	22.3	24.9	23.7	23.7	1.50	Lung	9.8	8.8	7.6	8.7	0.71
4 hr	Liver	26.3	25.9	24.3	25.5	1.35	Lung	5.4	6.3	5.8	5.8	0.61
8 hr	Liver	23.2	24.6	-	23.9	0.99	Lung	3.2	3.5	-	3.4	0.61

(Table I) There are also other potential sources of variability such as species differences and concurrent infections, but the characteristic curves of antimony concentration as a function of time are similar in comparable tissues

The whole blood curves (Figs 1 and 3) in all the experiments exhibit a high initial level which rapidly falls off, giving an exponential type of curve. One may note that the rate of disappearance of antimony from the chick blood is only about half as rapid as from the guinea pig blood

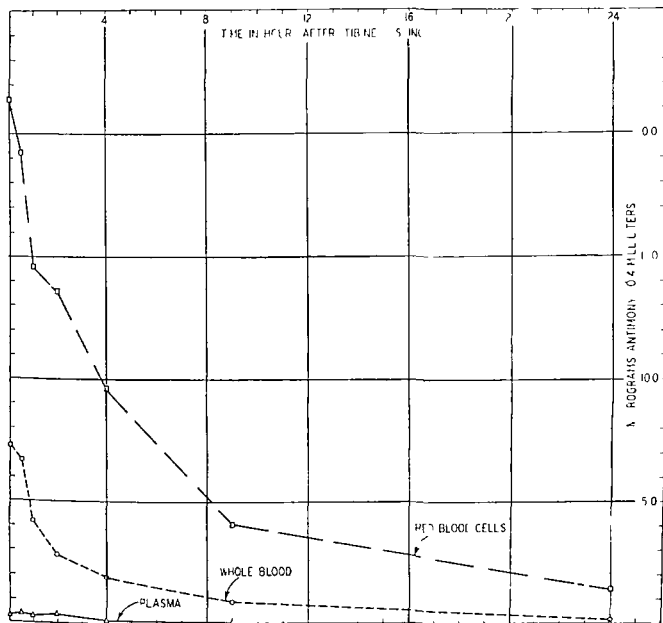


Fig 1—Antimony concentration in blood fractions of chicks infected with *P. gallinaceum*

The curves of the blood fractions are essentially similar in character to those of the whole blood (Fig 1). Although the red cell curve (Fig 1) is not corrected for packing, it is still evident that initially the antimony is largely held within the red cells rather than in the plasma. Later this partition is less evident and appears to approach a value of unity.

High doses of stibine are known to produce hemolysis of the red blood cells and this occurred to some extent in these experiments. Both the direct hematocrit readings on the whole blood and the values obtained indirectly by calculations from the blood fraction levels of antimony showed a progressive fall in

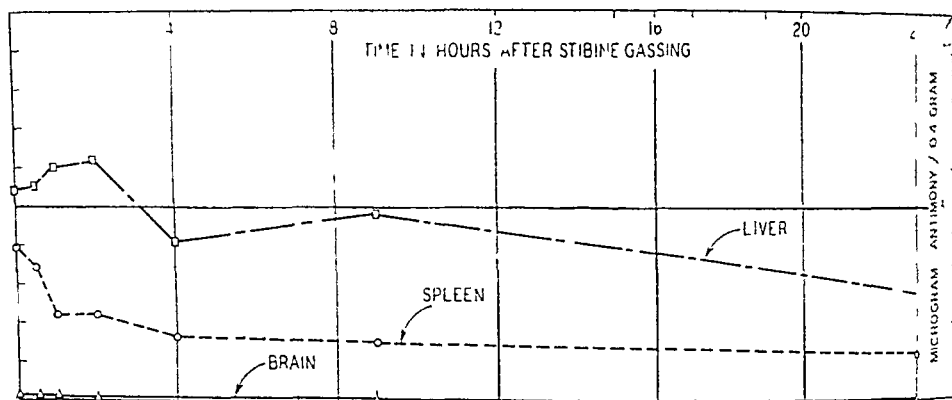


Fig 2 — Antimony concentration in liver spleen and brain of chicks infected with *P. gallinaceum*

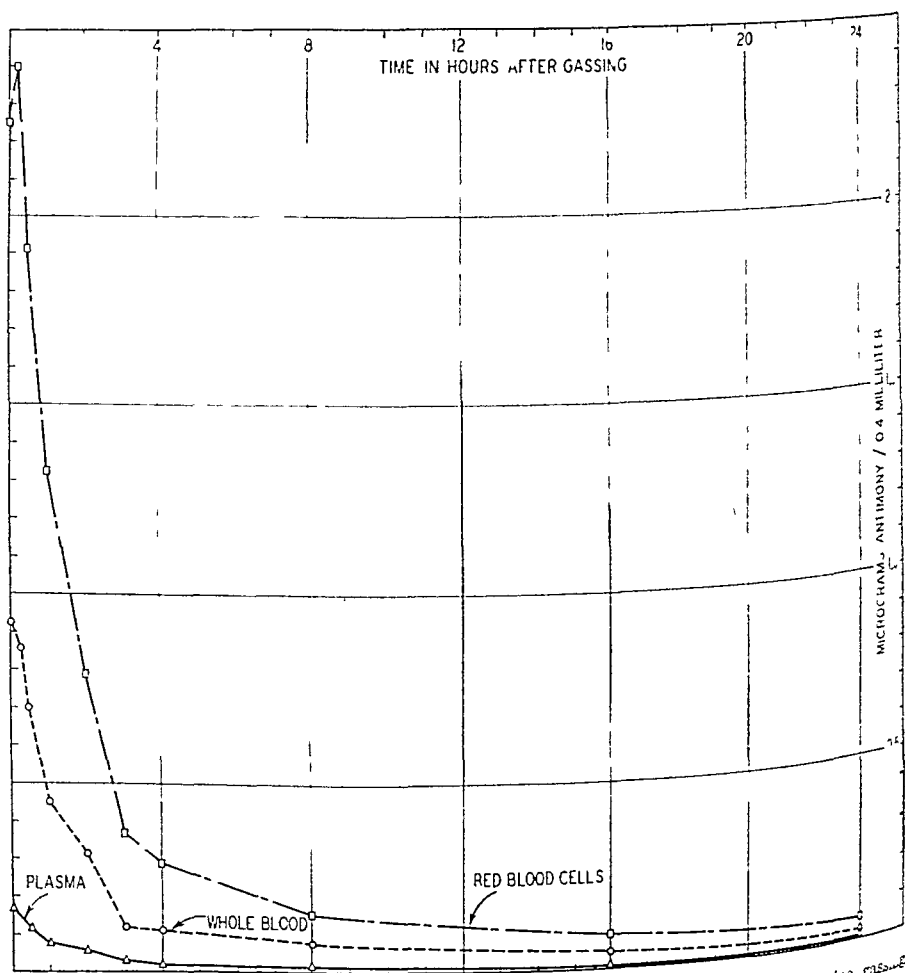


Fig 3 — Antimony concentration in blood fractions of guinea pigs following stibine gassing  
(Reproduced with the permission of the Naval Medical Bulletin)

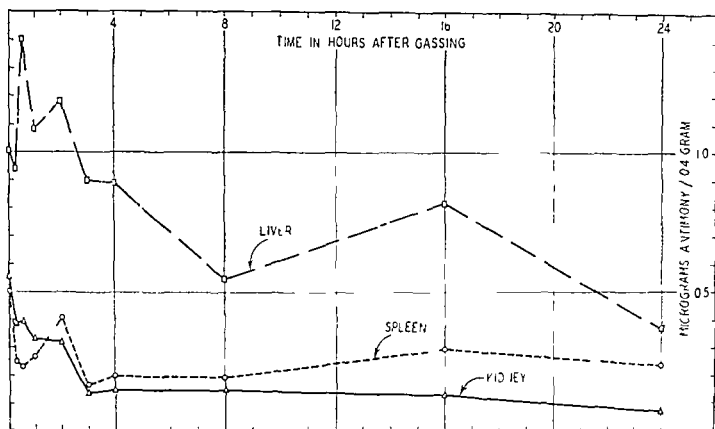


Fig 4—Antimony concentration in liver spleen and kidney of guinea pigs following stibine gassing

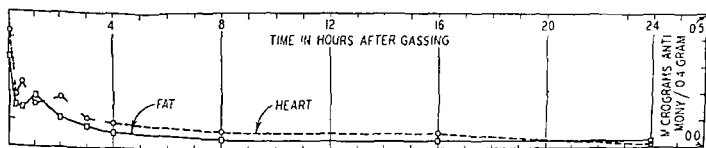


Fig 5—Antimony concentration in heart and perirenal fat of guinea pigs following stibine gassing

total red cell volume during the first hour following gassing amounting to about 30 per cent. After this the hematocrit remained constant at about 20 per cent red blood cells, whether the drop was due to hemolysis or not is uncertain.

From the present experiments the elimination curves of the lung, brain, muscle, and fat tissues (Figs 2 and 5) appear similar to those of blood, and the ratio of the antimony content of these tissues to that of whole blood remains fairly constant during the course of the elimination of antimony from the body.

The concentration in other tissues follows a somewhat different curve, of which perhaps the most obvious example is that of the liver (Figs 2 and 4). The result is the appearance of a maximum concentration, reached only after an hour or more. The spleen conforms in general to this same description. The curves for heart and kidney appear to fall in between the liver type and the blood type of exchange.

Some elementary insight into the dynamics of the biologic exchange of antimony may be gained from a study of the concentration ratio of antimony in the blood to that in the various tissues at successive times after the exposure

This is illustrated by a time plot of the ratios of tissue to blood concentration for the guinea pig (Fig 6). Data from the chick experiments yielded generally similar plots. Since the ordinate is logarithmic, it is easy to read the order of magnitude of the various ratios by inspection. For example, the liver concentration at four hours is eight times, while the plasma level is approximately one fifth that of whole blood. The slopes of the curves also furnish evidence as to

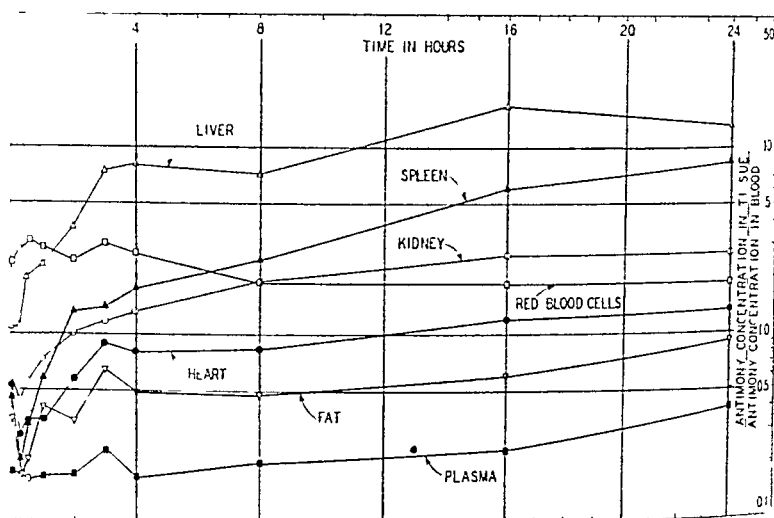


Fig 6—Ratios of antimony concentration in tissue to antimony concentration in blood (guinea pig) after stibine gassing

whether the elimination from any given organ is proceeding at a rate equal to (slope zero), greater than (slope negative), or less than (slope positive) that of the blood. For most of the data the terminal slopes appear not to depart much from zero. This is further supported by data pooled from several experiments (Table II) showing that the antimony concentrations of each of these

TABLE II AVERAGE PARTITION OF ANTIMONY BETWEEN TISSUES AND BLOOD IN CHICKS FOLLOWING TREATMENT WITH STIBINE, MEAN RATIOS (CONCENTRATION SB IN TISSUE/CONCENTRATION SB IN BLOOD)

TISSUE	HOURS AFTER GASSING				WEIGHTED GRAND AVERAGE
	8	16	24	52	
Liver	8.1 (4)*	12.0 (2)	9.1 (4)	7.4 (1)	9.11
Spleen	2.8 (3)	5.1 (2)	4.6 (3)	3.4 (1)	3.98
Kidney	2.1 (3)	1.7 (2)	1.9 (2)	1.6 (1)	1.89
Heart	0.64 (2)	0.85 (2)	0.90 (2)	0.65 (1)	0.70
Brain	0.017 (2)	0.014 (1)	0.018 (1)	----	0.01

\*Number of experiments in which the tissue-blood ratio was measured

tissues have attained a reasonably constant proportionality to blood at least by the end of eight hours.

With the foregoing in mind and with the knowledge that the blood curves (Figs 1 and 3) exhibit a very rapid initial drop, it is clear that during the

initial period the blood is actually losing its antimony simultaneously to nearly all other tissues of the body and its observed concentration is more a function of total blood flow and relative vascularity of organs than of excretion. During the later stages the loss by excretion comprises a far greater percentage of the blood loss than is the case initially. Certainly beyond three or four hours it appears that the blood elimination becomes approximately equal to that of most of the tissues, and the concentration ratios remain essentially at constant levels characteristic for different tissues. Thus as the grand means in Table II indicate after eight hours the highest concentration of antimony is found in the liver, followed by spleen, kidney, heart and brain in decreasing order. The concentration in the first three is always greater than in the blood; that in the heart is about equal to it and in the brain it is always about 1/100 of the whole blood level. A number of other tissues such as muscle and fat resemble brain in this respect and in their apparent tendency to approximate the plasma level of antimony concentration. This constancy in the tissue blood ratios for the chick and the guinea pig may have certain therapeutic implications, since after eight hours one might estimate the probable tissue antimony concentration from that of the blood alone.

In both the guinea pig and chick experiments the concentration of antimony in the bile was measured at the various time intervals and in the guinea pig that in the urine also. The curves for bile resembled those for liver but the peak concentrations were ordinarily higher than for liver and required about twice as much time to develop. Thus the liver peak in the chicks was always reached at from one to two hours post gassing while the biliary maximum occurred at around two and one half to five hours. In the guinea pig the maximum in liver was at one half hour and in the bile at two hours. The latter coincides with the time of the urinary maximum. Since in these experiments the total output of bile and urine was not known quantitative estimates of excretion by these routes cannot be made. However some information on the excretion of antimony administered as stibine may be obtained from the curve of the summated amounts in the various organs of the body as a function of time after the end of gassing calculated from the concentration of antimony and the volume of the respective tissue. Such data yield curves which have been fitted by inspection (Fig 7) and analyzed graphically. They give empirical equations expressing the elimination as a sum of decaying exponentials and their coefficients. Having obtained these expressions for the amounts present as a function of time, one may then differentiate to obtain the rate of elimination at any particular time. Thus at the end of the gassing period the guinea pig is found to be excreting antimony at an initial hourly rate of around 60 per cent of the total amount present while the chicks are much slower ranging from 6 to 30 per cent per hour. Otherwise stated it may be said that of the initial amount of antimony present at time zero the guinea pigs had excreted 50 per cent by the end of the first forty five minutes after gassing while the chicks required from two to five hours to reach the same degree of elimination. The rough correspondence between the curve of elimination of the body as a whole

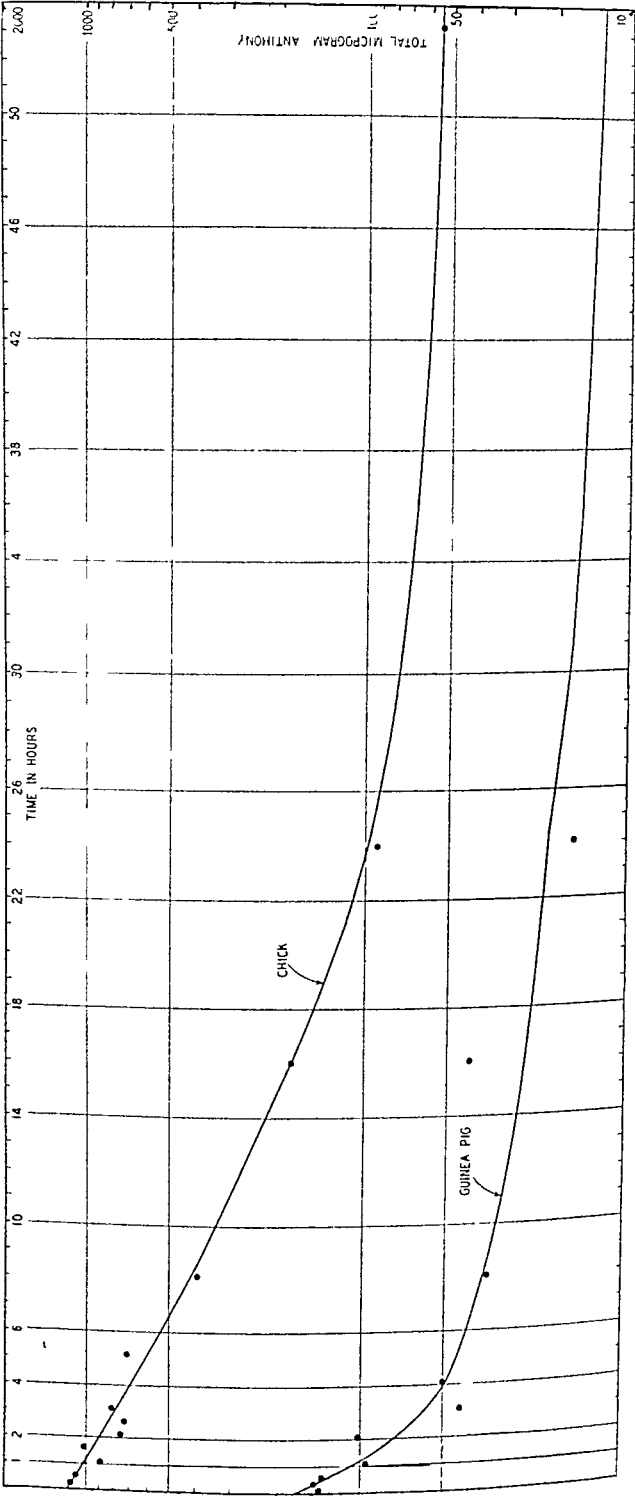


Fig. 7—Total measured antimony content of body following stibine gasping



(Fig 7) and the blood curve (Figs 1 and 3) appears to furnish additional evidence that, with stibine at least, a fair approximation to the elimination from the body may be had by recording the time curve of the blood after the first two hours have elapsed

The single experiment in which chicks heavily infected with *P gallinaceum* were gassed revealed no significant differences between these animals and the noninfected controls

#### SUMMARY AND CONCLUSIONS

Radioactive antimony administered as stibine gas ( $\text{SbH}_3$ ) to chicks (both normal and infected with *P gallinaceum*) and to normal guinea pigs has been measured in the blood and tissues at successive time intervals following its administration. Significant differences were not apparent between distributions in normal and infected groups

The concentration of antimony in the blood stream exhibited a smoothly decaying curve, decreasing more rapidly in the guinea pig than in the chick. The red cells contained initially a much higher concentration of antimony than did the plasma although this difference was reduced with time

The concentration curves of antimony in lung, brain, muscle and fat were generally similar to those of blood, while those of the liver and to a lesser extent of the spleen, passed through a maximum about one hour following treatment. Concentration curves of the kidney and heart were of variable shape

Approximately four hours after treatment the tissue antimony levels became constant with respect to order of rank: those in the liver, spleen and kidneys were greater than in whole blood; all other tissues showed concentrations less than the blood but as much as or more than the plasma

Evidence is adduced to show that the rate of elimination from the body is higher for the guinea pig than for the chick

In addition to the many who so generously assisted in the work from time to time we wish to acknowledge with particular gratitude the assistance of I. H. Gordon, PhM2, V6 USNR, Roy L. Evans, PhM2, V6 USNR, F. N. Cille, PhM2, V6 USNR and C. J. Spear, PhM1, V6, USNR

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# LABORATORY METHODS

## A SIMPLE BEDSIDE METHOD FOR THE ESTIMATION OF BLOOD SUGAR

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THE reaction between glucose and dinitrosalicylic acid for the estimation of glucose was first used by Sumner in 1921<sup>1</sup> Since then, the Sumner reagent has been in general use for the measurement of urine glucose and recently has been adapted for the determination of blood glucose on a tungstic acid filtrate.<sup>2</sup>

The following procedure is an extremely simple method for the approximate determination of blood sugar on a zinc hydroxide filtrate using the Sumner reagent as modified by Epton.<sup>3</sup> The method, although adaptable within certain limits for a strictly quantitative procedure, as discussed later, is primarily designed for an approximate estimation of blood sugar by the physician or nurse with a minimum of time and equipment and limited laboratory facilities. As shown in Table I, the approximate results obtained in five minutes using the color standards or color chart described here are essential checks with determinations as carried out by the Folin-Wu<sup>4</sup> macromethod and the Folin Malmros or the rapid Hagedorn-Halstrom-Jensen<sup>5</sup> micromethods.

### REAGENTS

(A) 10 Per cent zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ )

(B) 0.5 N Sodium hydroxide (A stock solution preferably is kept in a paraffined bottle.)

These solutions, A and B, are the regularly employed macrosolutions for preparing a Somogyi blood filtrate.<sup>7</sup> They may be conveniently kept in small bottles provided with dropping pipettes graduated to deliver 0.4 ml of solution. Stock bottles, tightly stoppered, keep well over a period of months.

(C) A solution of dinitrosalicylic acid prepared as follows.<sup>3</sup>

(1) A sodium potassium phenol stock solution is made by dissolving 400 Gm of Rochelle salts (sodium potassium tartrate) in about 600 ml of warm distilled water and adding 13 Gm of phenol crystals. The Rochelle salts and the phenol are dissolved and mixed well, and the solution is then diluted to a volume of 1,000 milliliters.

(2) 12 Gm of monosodium 3,5 dinitrosalicylate\* are dissolved in 700 ml of distilled water heated to a temperature of 65° C. When dissolved, the entire volume is poured into the liter of sodium-potassium phenol stock solution and mixed well. A yellow precipitate forms.

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\*Eastman Kodak Company, Rochester, N. Y.

(3) 6 Gm of sodium bisulfite ( $\text{NaHSO}_3$ ) are added to the solution (2) and the whole is thoroughly mixed

(4) Finally, 300 ml of 10 per cent sodium hydroxide (25 N) are added and the final solution is completely mixed. The yellow precipitate formed in step 2 disappears at this point. The solution should be put in a brown bottle and allowed to stand for about one week before using.

To test the solution titrate 5 ml with normal acetic acid to the appearance of a white precipitate. If the reagent has been correctly prepared 1.86 ml of the acid should be required for 5 ml of the reagent. This solution, if kept in the dark in a brown bottle, is stable for at least four to five months. If any cloudiness develops, the solution should be filtered before use.

#### APPARATUS

(A) A test tube approximately 12.5 cm long and 15 mm in diameter graduated at 3.8 ml, Tube 1

(B) A Pyrex test tube approximately 15 cm long and 15 mm in diameter, graduated at 2.0 and 4.0 ml, Tube 2

(C) A cup or beaker in which a small amount of water may be boiled

(D) A small funnel fitted with a piece of folded filter paper (Whatman No. 1, 5.5 or 7.0 cm in diameter)

(E) A micro blood pipette graduated at 0.1 and 0.2 milliliter

(F) A prepared color chart or a series of color standards

#### PROCEDURE

Measure into Tube 1, 0.4 ml each of Reagents A and B using the calibrated droppers with which the bottles are equipped. Add distilled water to the 3.8 ml mark. In all quantitative measuring it should be remembered always to use just sufficient liquid to make the bottom of the meniscus on a line with the mark calibrated for the desired amount. Rotate the tube gently and introduce into the precipitating mixture exactly 0.2 ml blood taken directly from the ear or finger by means of a micro blood pipette. (Venous blood may be used if collected with fluoride oxalate\* as a preservative and anticoagulant.) Mix the contents of the tube with the pipette by alternately sucking up and blowing out the blood mixture then filter immediately into Tube 2 by means of the small funnel and filter paper. A water clear filtrate should result†. Allow the filtrate to collect exactly to the 2 ml mark. We have found that by using the technique described with Whatman No. 1 filter paper 5.5 or 7.0 cm in diameter 2 ml of filtrate may be secured easily and rapidly. Add Reagent C to the 4 ml mark. Mix thoroughly by rotation and immerse in a boiling water bath for exactly three minutes. The color changes during the heating period from a pure yellow to

\*The fluoride oxalate is prepared by thoroughly mixing together 0.0 Gm powdered sodium fluoride and 0.0 Gm powdered potassium oxalate. Seventy milligrams of the mixture are used for each 5 ml of blood.

†Although for complete precipitation of protein a freshly precipitated zinc hydroxide solution is not usually recommended unless heat is used we have found that the double concentration of reagents yields a very clear filtrate which gives an essentially negative biuret reaction.

TABLE I COMPARISON OF BLOOD SUGAR VALUES, MG PER 100 ML, BY DIFFERENT METHODS

SAMPLE	AUTHORS' RAPID METHOD		FOLIN WU <sup>4</sup> (MACRO)	FOLIN MALMROS <sup>5</sup> (MICRO)	HAGEDORN HALSTROM JENSEN <sup>6</sup> (MICRO)
	VISUAL	PHOTOELECTRIC			
1	300*			300	302
2	400*			400	
3	120	404			119
4	150	162	114	152	158
5	200	208		194	211
6	255	266	253	266	
7	220	225	218	228	224
8	250	258		248	252
9	125			129	127
10	100			105	90
11	290			278	289
12	100			97	94
13	200	208	200	198	
14	200		186		183
15	175	185	178		189
16	250*			244	
17	350			336	
18	50*			58	
19	200	208	191		193
20	225		195		
21	110				102
22	210		191		
23	160		157		
24	105			94	
25	110			105	
26	100			101	
27	110		97		
28	115			112	
29	102			94	
30	150	152	143	142	152
31	250	236	226	228	250
32	175	173			172
33	200	192		186	199
34	175	172	172		172
35	190	202	203	200	
36	105*	108		103	
37	580		630		
38	290†			270†	
39	265†			252†	
40	320†			338†	
41	190		178	180	
42	165		165		
43	155		141	148	
44	200†			206†	
45	100†			94†	
46	105†			92†	
47	100†			96†	
48	95		94		
49	105		102		
50	90		98		
51	110		114		
52	110	110	111	105	228
53	220	225		218	376
54	390	374			
55	100	87	90	85	
56	120	120	121	116	
57	100	97	90	85	
58	125	132	128	135	
59	110	101		103	
60	130	123		119	

\*Comparison made with color chart alone

†Different capillary blood samples obtained simultaneously from the ear

a deeper yellow, yellow brown, or reddish brown depending on the concentration of glucose present. Cool slightly after removal from the water bath and compare the color obtained with the color chart or standard tubes, matching it to the nearest color. Shades between standards can be approximated. Table I gives the results obtained on sixty blood samples using the described rapid estimation in comparison with other standard methods. In each case the rapid approximation was done either before the accurate determinations or was done by a second individual who had no knowledge of the correct results obtained with standard procedures.

#### PREPARATION OF COLOR STANDARDS

A series of accurate color standards may be prepared as outlined below. If prepared under the conditions given these standards are accurate to within 5 per cent for a period of at least one month if kept in the refrigerator (40° F.) when not in use.

An accurate stock glucose solution prepared in saturated benzoic acid containing 0.2 Gm glucose per 100 ml is required. This solution is permanently stable if kept free from contamination. A dilute standard containing 10 ml of this stock diluted to 100 ml with distilled water is used in preparing the standard tubes as follows.

TABLE II CHART FOR PREPARATION OF GLUCOSE STANDARDS

DILUTE GLUCOSE STANDARD (ML)	BOILED WATER (ML)	BLOOD SUGAR EQUIVALENT (MG PER 100 ML)
0.5	3.5	50
1.0	3.0	100
1.5	2.5	150
2.0	2.0	200
2.5	1.5	250
3.0	1.0	300

A series of tubes using quantities of the *dilute* glucose standard in increasing amounts of 0.5 ml from 0.5 to 2.5 or 3 ml is prepared. Freshly boiled distilled water which has been cooled is added in amounts to make a total volume of 4 milliliters. Then to each tube exactly 4 ml of Reagent C are added, the contents of the tubes are mixed and the tubes placed in a boiling water bath for three minutes as outlined under Procedure for blood. After cooling 4 ml of the colored solutions are transferred to clean, dry tubes of the same diameter as those in which the determination is to be made. Two drops of toluol are added to each tube and the tubes stoppered and sealed with paraffin and labeled with the proper value in milligrams per 100 ml of blood.

The series of standards which we have used is given in Table II. Standards between these values may be prepared but are not necessary. It is advisable however to prepare a new series of standard tubes with every new lot of Reagent C as slight variations may occur.

Although the series of color standards may be preferable a color chart from these standards may be prepared with ordinary paints. With a little experience good approximations can be made with the color chart (Table I). It was not feasible to reproduce here the color charts made and used by us.

## DISCUSSION

The validity of the foregoing procedure as a rapid method for the estimation of blood sugar is apparent from the results given in Table I. Certain points regarding the method, however, should be noted. The reduction of the Sumner reagent, under the conditions outlined, is directly proportional to the concentration of glucose present between the equivalent of a blood sugar of 75 mg and 300 mg per cent, Fig 1.

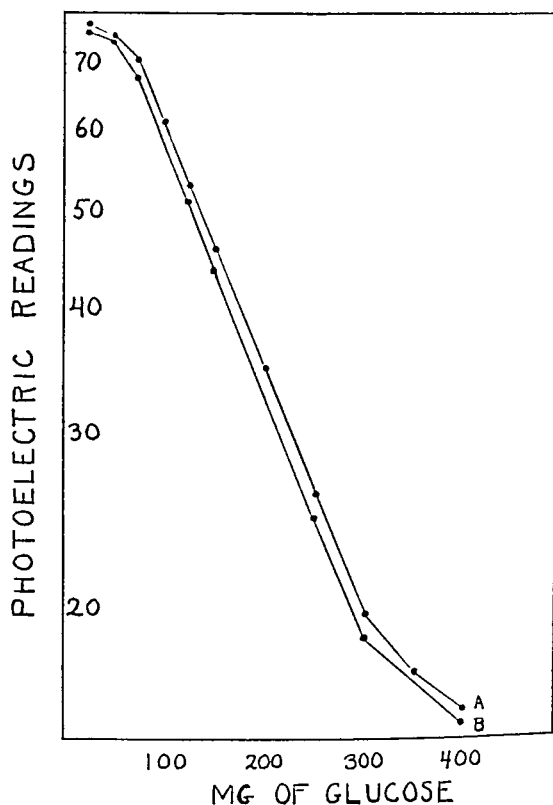


Fig 1—Two typical curves obtained from two series of standard glucose solutions using different lots of Reagent C. Curve A was done July 17, curve B December 15. The readings were taken on a Leitz photoelectric colorimeter using the green filter No. 401 and plotted against the glucose concentration expressed as milligrams of blood sugar per 100 milliliter.

The curves given in Fig 1 were obtained with standard glucose solutions using a Leitz photoelectric colorimeter with the green filter, Leitz No. 401. Slight variations occur, as indicated by these two typical curves, with different lots of Reagent C. It is essential, therefore, if the color of an unknown is to be measured accurately on a previously standardized photoelectric colorimeter, that the instrument be restandardized with each new lot of Reagent C. Although quantitative technique throughout the procedure would obviously be desirable if the final measurement of color was exact, our results do not warrant such an assumption as a necessity. With accurately calibrated tubes and reasonably careful technique, any error due to lack of quantitative pipetting is not of clinical significance, Table III.

TABLE III COMPARISON OF BLOOD SUGAR VALUES WITH AND WITHOUT QUANTITATIVE MEASUREMENT, MG PER 100 ML

SAMPLE	TECHNIQUE AS RECOMMENDED		QUANTITATIVE MEASURING		FOLIN MALMRO'S OR FOLIN WU <sup>4</sup>
	VISUAL	PHOTOELECTRIC	VISUAL	PHOTOELECTRIC	
1	110	110	100	100	105
2	120	120	110	117	116
3	100	97	105	105	90
4	125	132	120	130	135
5	150		150	157	150
6	200		200	213	202
7	150		150	163	160

In the procedure as recommended the precipitation of blood protein is effected by means of freshly precipitated zinc hydroxide. The concentration of zinc sulfate and sodium hydroxide used is sufficient to yield a protein free filtrate without the use of heat\*. The filtration is rapid and the necessary volume of filtrate is quickly and easily obtained. A second procedure for precipitation of the protein may be used if desired. The blood may be pipetted directly into 3 ml of water the pipette being rinsed thoroughly by alternate sucking up and blowing out of the water solution. To the blood and water solution then the necessary quantities of Reagents A and B are added the tube is stoppered and the contents are mixed by shaking well. Using this method for precipitation of the protein filtration is less rapid and the total volume of filtrate is slightly less. If this method of precipitation is adopted, it is recommended that tubes graduated at 1.5 and 3.0 ml be used rather than at 2.0 and 4.0 milliliters.

TABLE IV COMPARISON OF BLOOD SUGAR VALUES OBTAINED WITH DIFFERENT TECHNIQUES FOR PRECIPITATION OF PROTEIN MG PER 100 ML

SAMPLE	TECHNIQUE A*		TECHNIQUE B†		FOLIN MALMRO'S OR FOLIN WU <sup>4</sup>
	VISUAL	PHOTOELECTRIC	VISUAL	PHOTOELECTRIC	
1	100	90	75	76	81
2	115	109	115	109	101
3	110	111	95	87	105
4	95	100	80	77	85
5	120	123	110	125	112
6	220	224	200	187	218
7	390	374	385	366	376
8	105	104	95	93	99

\*Blood introduced into Zn(OH)<sub>2</sub>

†Blood introduced into water then ZnSO<sub>4</sub> solution (Reagent A) added followed by NaOH solution (Reagent B)

This method for precipitation of the protein offers the advantage of not getting the protein precipitate in the micropipette thus making the cleaning of the pipette considerably easier. Furthermore this method for precipitation of the protein yields a true Somogyi filtrate and in some instances the blood sugar values obtained may be appreciably lower (Table IV) approaching probably the true blood sugar value.

Since in its simplest form the method is dependent for the final estimation on a color comparison with standard tubes or a color chart the importance of

standardizing one's individual technique for judging the color cannot be over emphasized. If standard tubes are used, the unknown solution should be in the same size tube as the standards. In using the standard tubes, we have obtained the best results by holding the unknown tube between the two closest standards and judging the light which comes through the tubes. In this way the density of the color can be fairly accurately approximated. If a color chart is used, we have obtained the best results by holding the tube containing the unknown obliquely above the block of color nearest in shade, with the source of light coming toward the tube from behind the worker. We have used daylight for all of our comparisons.

The determination is designed for 0.2 ml of blood. When a blood sugar value over 300 mg per cent is found, greater accuracy can be obtained by repeating the determination using 0.1 ml of blood and subsequently multiplying the result by two. In this way, comparison is made with a standard under 300 mg rather than by an attempt at a comparison with the very deep red brown colors obtained with a 400 or 500 mg standard.

The method in its present form is not suitable for the estimation of very low blood sugar values. Between 50 and 75 mg per cent, fairly good approximations can be made. Below 50 mg per cent the color of the reagent blank is so intense that any increase of color due to glucose is indiscernible.

#### SUMMARY

A simple method designed for the rapid estimation of blood sugar by the physician or worker with a minimum of laboratory experience and equipment has been described. The method is based on the reduction by glucose of dimethylsalicylic acid and closely checks determinations done by other standard methods.

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#### Erratum

In the paper by Maltaner and Gnesch, A Method for the Determination of Titers Between 10 and 100 in the Quantitative Complement Fixation Test for Syphilis, which appeared in the March issue of the JOURNAL (33: 383, 1948) in the column heading of Table II, p. 384, 1<sup>st</sup> column should read 12.5.



# PRODUCTION OF TEMPORARY DIABETES MELLITUS IN MAN WITH PITUITARY ADRENOCORTICOTROPIC HORMONE RELATION TO URIC ACID METABOLISM

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THE production in normal animals of either temporary or permanent diabetes by the injection of crude saline extracts of anterior pituitary gland has been amply demonstrated<sup>1, 3</sup> and repeatedly confirmed<sup>4</sup>. The factor or factors responsible for the diabetogenic activity of these extracts remain unknown. Using biologically pure growth hormone Marx, Anderson, Fong and Evans reported an increase in glycosuria of partially depancreatized rats. Ingle, Li and Evans<sup>5</sup> used 7 mg. per day of pure adrenocorticotrophic hormone (A.C.T.H.) in normal rats force fed a high carbohydrate diet and they observed increased urinary nitrogen, glycosuria and hyperglycemia during the period of administration. Bennett and Li<sup>7</sup> produced marked increase in glycosuria and severe nitrogen loss in alloxan diabetic rats with the use of pure adrenocorticotrophic hormone. When they used pure growth hormone in such preparations they observed only an occasional increase in glycosuria. It is significant that this occurred even in the presence of the nitrogen retention which characterizes the activity of growth hormone. Thus so far as pure pituitary fractions are concerned, adrenocorticotrophic hormone has been found to have the greatest diabetogenic effect of any tested to date. Since it is known too that administration of suitable amounts of C11 or C11-17 oxy steroids to rats is diabetogenic<sup>8, 10</sup> the assumption is valid that the diabetogenic activity of adrenocorticotrophic hormone is due to cortical elaboration of large amounts of C11 and C11-17 oxy steroids among other possibilities.

In man neither a diabetogenic nor an anti-insulin effect of administered corticosteroids has been demonstrated<sup>11, 12</sup> with doses as high as 100 mg. per day of 11-dehydrocorticosterone. This may be the result of too small a dose if the estimation of Ingle and co-workers<sup>6</sup> of the secretory potentiality of the adrenal gland is valid.

The administration of purified adrenocorticotrophic hormone to man has been limited by the difficulties attending its preparation. It was used first in two normal men by Browne<sup>13</sup>. No spontaneous glycosuria was observed in either subject. One who had received 40 mg. per day for two days showed a decrease in carbohydrate tolerance on the morning of the third day and glycosuria was noted during the glucose tolerance test (100 Gm. orally). Thirty

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minutes before the test the subject had received 40 mg of adrenocorticotrophic hormone subcutaneously. In two recent reports of experiments in which larger doses were given, no glycosuria was observed. Mason and co-workers,<sup>14</sup> employing doses as high as 100 mg per day (eight injection days over a twelve day period) of adrenocorticotrophic hormone (prepared according to the method of Li, Evans, and Simpson<sup>15</sup>), found no impairment of carbohydrate tolerance but observed a mild increase in resistance to insulin. Foisham and co-workers<sup>1</sup> using 40 mg per day of adrenocorticotrophic hormone, prepared by a modification of the method of Sayers and associates,<sup>17</sup> for six days, noted a small rise of the fasting blood sugar level in their normal subject. No glycosuria occurred. Glucose tolerance tests were not reported.

The present report deals with the production of a diabetic state in all of three normal subjects who received daily injections of purified adrenocorticotrophic hormone. This state was characterized by glycosuria and a significant loss of carbohydrate tolerance during the entire period of adrenocorticotrophic hormone administration. In two of these subjects, one man and one woman, the loss of tolerance for carbohydrate was great. These subjects exhibited severe negative nitrogen balance during the period of adrenocorticotrophic hormone injection. The third subject, a woman, developed a milder glycosuria, lost much less tolerance for carbohydrate, and demonstrated no negativity in the nitrogen balance study. In fact, glycosuria developed during a period of nitrogen retention.

#### METHOD OF STUDY

*Procedure*—Two apparently normal, 24 year old, female senior medical students (subjects A M and M W) were fed a constant, carefully weighed diet for thirty two consecutive days. The diet contained 99 Gm of protein, 300 Gm of carbohydrate, and 176 Gm of fat. Analysis showed nitrogen, 15.75 Gm, sodium, 4.40 Gm, and potassium, 4.8 grams.

The first twelve days established a reliable base line. During the next eight days each subject received 120 mg of adrenocorticotrophic hormone (Batch 37 K E, Table I) intramuscularly daily. The total amount was given in three doses (40 mg every eight hours). The remaining twelve days were used to study recovery from the abnormal metabolism induced.

The third normal subject, a 37 year old dental student (Subject R S), was fed a constant diet for thirty three days. It contained 99 Gm of protein, 300 Gm of carbohydrate, and 197 Gm of fat. Analysis showed nitrogen, 15.75 Gm, sodium, 4.63 Gm, and potassium, 4.86 grams. After a ten day base line period the subject was given 50 mg of adrenocorticotrophic hormone every eight hours (150 mg per day) intramuscularly daily for the next ten days. This material was from Batch 37 K G (Table I). During the last thirteen days the recovery phase was studied.

*Pituitary Adrenocorticotrophic Hormone*—The materials used in this study were prepared by a modification of the method of Sayers and co-workers.<sup>14</sup> The laboratory has upheld us with the properties of these materials (Table I). In all cases the adrenocorticotrophic hormone was given in a concentration of 10 mg per 1 cc of saline.

*Chemical Methods*—Determinations performed, which are connected with this report, were blood sugar,<sup>18</sup> blood uric acid,<sup>19</sup> blood glutathione,<sup>2</sup> urinary uric acid,<sup>1</sup> and urinary glucose.<sup>22</sup> All nitrogen determinations were done by the macrokjeldahl method. Glucose tolerance tests performed during the period of adrenocorticotrophic hormone administration were begun at 8 A M, nine hours after the last injection of adrenocorticotrophic hormone.

\*We are indebted to Dr J R Mote. The Armour Laboratories Chicago Ill for the purified adrenocorticotrophic hormone used in these studies.  
†In the Armour Laboratories Chicago Ill

order to avoid any immediate or extraneous glycogenolytic effect which could be assigned to the adrenocorticotrophic material used. On such days the dose of adrenocorticotrophic hormone usually given at 7 A.M. was given at the conclusion of the test (noon). The glucose was administered orally on the basis of 1.75 Gm. per kilogram of base line body weight, and the same total amount was given for all subsequent tests (Subject M.W. 108 Gm., Subject A.M., 104 Gm., Subject R.S., 122 Gm.)

TABLE I PROPERTIES OF THE ADRENOCORTICOTROPIC HORMONE PREPARATIONS EMPLOYED

BATCH	A C T H ACTIVITY (PER CENT OF ARMOUR STANDARD*)	PRESSOR ACTIVITY— ROOSTER B P (UNITS/MG)	OXYTOIC ACTIVITY— GUINEA PIG UTERINE STIMP (UNITS/MG)	PROLACTIN ACTIVITY— PIGEON CORP. SAC (UNITS/MG)	GOVADOTROPIC ACTIVITY— COLLIP (UNITS/MG)	SOLUBILITY
7 K E	41.5 ± 12	0.005	0.0025	0.5	2.0	Soluble in saline
37 K G	34.8 ± 2	0.066	0.017	0.5	2.0	Soluble in saline

The biologic activity of Armour A C T H Standard (LA 1 A) is such that a single intravenous injection of 0.004 mg. produces consistently a 0 to 30 per cent decrease in adrenal ascorbic acid content of the hypophysectomized rat.

## RESULTS AND COMMENTS

No immediate symptomatic effects of injections of adrenocorticotrophic hormone were experienced by any of the subjects. This is different from the experience of Forsham and co-workers<sup>1</sup> who used different batches of Armour's purified adrenocorticotrophic hormone and observed symptoms suggesting posterior pituitary pressor and oxytocic activities. Subject M.W. developed redness and itching at the site of injections during the last three days of administration (Days 6, 7, and 8). More annoying to her at this time was the itching and discomfort which occurred at sites of prior injections. Subject R.S. developed severe acne of the face, scalp, shoulders, back, chest and abdomen on the eighth day of injection. He had never had acne. This began to regress slowly upon cessation of injections (eleventh day) but persisted with continued regression for four weeks. Both female subjects experienced gross irregularity of the menstrual cycle which followed the adrenocorticotrophic hormone injection period. The next cycle returned to normal. It should be mentioned that in all three subjects there occurred a four to fivefold increase over the base line of the daily total excretion of 17 ketosteroids.<sup>3</sup> This is a much greater increase than has been observed previously<sup>13,1</sup> but the amounts of adrenocorticotrophic hormone employed in our experiments were greater than these previously used. A great retention of sodium and a diuresis of potassium were also observed in all subjects.<sup>23</sup>

Table II and Fig. 1 demonstrate the data bearing on carbohydrate metabolism which were obtained on Subject R.S. This subject showed the most striking disturbance of sugar metabolism of all three subjects given adrenocorticotrophic hormone. During the ten day adrenocorticotrophic hormone period a total of 252 Gm. of glucose was excreted in the urine, 12 Gm. appearing on the first day and 36 and 33 Gm., respectively, on each of the last two days. During the same period, urinary nitrogen averaged 19.3 Gm. per day as compared with an

average of 13.5 Gm per day observed in the base line period. A total of 36.7 Gm of extra nitrogen was excreted in the urine in the adrenocorticotrophic hormone period. This represents loss of 360 Gm of body protein in ten days, or 36 Gm per day, despite a daily intake of 99 Gm of protein. Negative nitrogen balance continued for ten days after cessation of adrenocorticotrophic hormone and amounted to an additional loss of 119 Gm of body protein.

If, for the adrenocorticotrophic hormone period, one assumes that all of the excreted glucose had arisen by excessive glycconeogenesis from protein one arrives at a D/N ratio of 4.3 and a glucose from protein conversion rate of 70 per cent. That such an assumption is not justified under the conditions of this

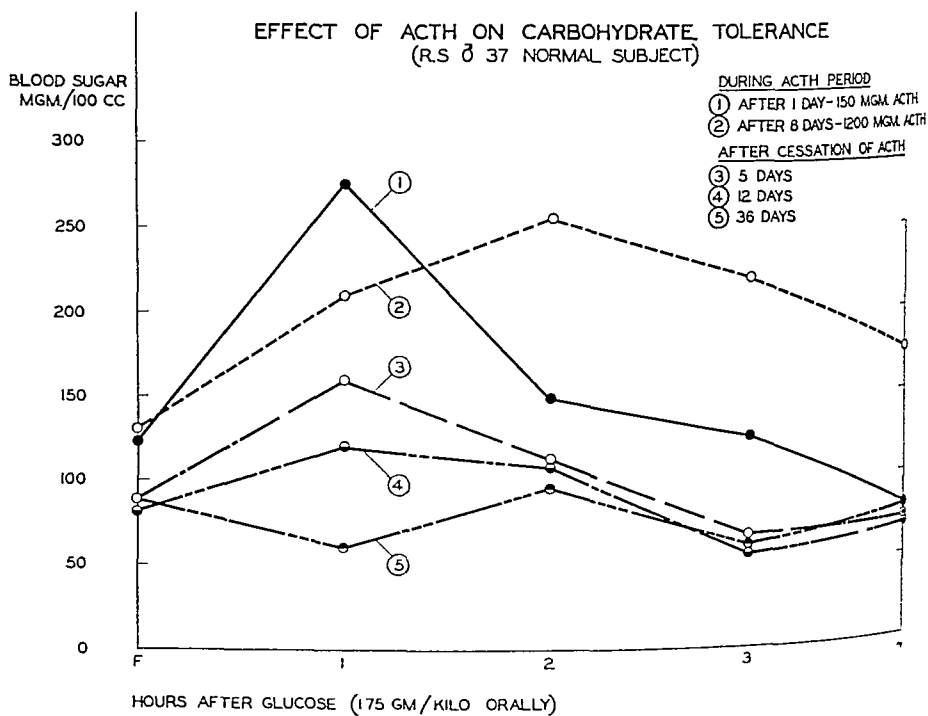


Fig 1

study is obvious. There is no reason to believe that the subject could not have disposed of an additional 100 or more grams of glucose (over and above the 300 Gm contained in his diet) normally and without glycosuria had he not been receiving adrenocorticotrophic hormone. To assume, then, that glucose derived from body protein so taxes the normal mechanisms by which glucose is utilized that those mechanisms break down is unreasonable, and especially so when one realizes that an average of 36 Gm of sugar per day is the maximal amount that could have been derived from protein, in Subject R. S., had 100 per cent conversion been possible. Thus it becomes clear that regardless of the source of the glucose (dietary carbohydrate and protein, and endogenous protein), there has occurred a very important over-all decrease in the capacity of the subject

TABLE II EFFECT OF INTRAMUSCULAR ADMINISTRATION OF ADRENOCORTICOTROPIC HORMONE UPON CARBOHYDRATE, NITROGEN AND URIC ACID METABOLISM (NORMAL MALE SUBJECT R S 37 YR)

DAY	ACTH (MG DAY)	URINARY GLUCOSE (GM / DAY)	FASTING BLOOD SUGAR (MG / 100 CC)	URINARY NITROGEN (GM / DAY)	URINARY URIC ACID (MG / DAY)		BLOOD URIC ACID (MG / 100 CC)	BLOOD GLUTA THIONE (MG / 100 CC)
					A	B		
1	0	0		14.0	464	52		
2	0	0		14.1	560	680		
3	0	0		1.6	16	679		
4	0	0	84	1.7	492	667		
5	0	0		12.7	4.4	5.6	1	40.0
6	0	0		13.2	124	1.0		
7	0	0	84	13.1	13	27	0	1.8
8	0	0		14.4	536	56		38.6
9	0	0		1.3	2.4	3		
10	0	0		10.1	824	5		
11	150	12	83	16.9	808	101	3.1	5.0
12	150	20	122	17.2	160	948	4.9	30.0
13	150	21	112	19.0	800	896	2.3	30.2
14	150	25		13.4	828	1001		
15	150	21	108	20.1	124	926		
16	150	24		20.3	780	1034		
17	150	25	124	11.1	760	1014		
18	150	36	129	21.7	86	140		
19	150	33	128	20.8	9.0	1153	2.4	21.2
20	0	3		17.0	688	642		
21	0	0		14.5	864	1012		
22	0	0		13.7	824	788		
23	0	0		14.7	760	614		
24	0	0	66	14.9	732	660		
25	0	0	86	14.7	616	619		
26	0	0		14.8	560	556		
27	0	0	76	14.2	692	570	3.2	32.8
28	0	0		13.9	604	523		
29	0	0		13.9	588	556		
30	0	0		12.8	623	509		
31	0	0		11.8	508	487		
32	0	0	82	11.1	444	483		

†4.

Before first injection

†Day of glucose tolerance test (see Fig. 1)

 A Method of Benedict and Franke<sup>2</sup> B method of Buchanan and co worker<sup>22</sup>

to dispose of glucose normally (by oxidation and/or conversion to fat). This contention is supported by the facts that not only has nonutilized glucose appeared in the urine but, in addition, more unutilized glucose has accumulated in the body fluids (see Table II Fasting Blood Sugar Levels), the liver,<sup>1</sup> and very likely in the muscles as well. Further, that this state of affairs (decreased capacity to utilize glucose) is related not to negative nitrogen balance but to the presence of adrenocorticotrophic hormone activity is evident (compare Days 11 and 12 with Days 21 and 22). Finally, the glucose tolerance curves (Fig. 1) demonstrate a greatly decreased capacity to clear the blood of absorbing glucose. This deficiency is apparent after one day of adrenocorticotrophic hormone administration. After eight days of adrenocorticotrophic hormone there can be little question that a diabetic state is present.

In the light of present knowledge two main possibilities exist by which one might explain the decreased capacity of the tissues to dispose of glucose

(1) Interference with the peripheral activity of insulin due to (a) increased hexokinase inhibition by corticosteroids or by adrenocorticotrophic hormone, (b) stimulation of pancreatic alpha cell secretion<sup>24</sup> by either adrenocorticotrophic hormone or adrenal corticosteroids, and/or (c) destruction of insulin peripherally

(2) Diminution in the production and/or release of insulin from the beta cells of the pancreatic islets

No attempt was made to study the first possibility. The second one was studied with respect to uric acid metabolism and the blood levels of glutathione (Table I)

The administration of adrenocorticotrophic hormone produces a large increase in the urinary excretion of uric acid accompanied by a decrease of blood uric acid. It is believed by some<sup>15</sup> that neither increased blood clearance for uric acid nor hemodilution can adequately explain these phenomena and that increased production of uric acid probably accounts for the increased excretion. It is conceivable that during the increased production of uric acid which occurs as the result of administration adrenocorticotrophic hormone (and which is induced by the elaboration of excessive amounts of 11 and 11-17 oxygenated cortical steroids), intermediaries, similar in their effects to alloxan are produced and reach a critical intracellular concentration in the beta cells. Such a concept need not imply necrosis of beta cells, as is known to occur after the administration of large amounts of alloxan, but could suggest depression of intracellular enzymatic production of insulin as the result of prolonged exposure to relatively lower concentrations of noxious intermediaries of uric acid metabolism.

It is appreciated that such considerations must be regarded as speculative at present. But in Table I are recorded data related to the problem. Note that the lowest levels for blood glutathione were obtained during the adrenocorticotrophic hormone period (determinations of glutathione were not done in the other two subjects). Note, too, that the highest levels of urinary uric acid were obtained during the same period. It is well known that administration of alloxan to animals produces a prompt and severe depression of the level of blood glutathione<sup>25</sup>. The recent report of Griffiths<sup>26</sup> showing the production of hyperglycemia in rabbits by first lowering blood glutathione by means of a diet deficient in cystine and methionine and then administering uric acid intraperitoneally suggests a possible relationship of those experiments to the ones here reported.

The results of the studies of carbohydrate metabolism in the other two adrenocorticotrophic hormone treated subjects are shown in Table III and Figures 2 and 3. Subject A. M., while ingesting 99 Gm of protein daily, destroyed, in addition, 113 Gm of body protein during the eight-day adrenocorticotrophic hormone period, an average of 14 Gm per day. A total of 30 Gm of glucose appeared in the urine over the same interval. From the fasting blood sugar level and the daily glycosuria (Table III) it appears that in this subject the me-

E III EFFECT OF INTRAMUSCULAR ADMINISTRATION OF ADRENOCORTICOTROPIC HORMONE UPON CARBOHYDRATE NITROGEN, AND URIC ACID METABOLISM (TWO NORMAL FEMALE SUBJECTS)

A C T H (MG / DAY)	A M 24 YR				M W 24 YR			
	URINARY GLUCOSE (GM / DAY)	FASTING BLOOD SUGAR (MG / 100 C C)	URINARY NITROGEN (CM /DAY)	URINARY URIC ACID (MG /DAY)	URINARY GLUCOSE (CM /DAY)	FASTING BLOOD SUGAR (MG / 100 C C)	URINARY NITROGEN (GM /DAY)	URINARY URIC ACID (MG /DAY)
0	0		81	440	0		11.2	490
0	0		105	570	0		11.2	500
0	0		102	490	0		11.4	450
0	0		123	520	0		11.4	450
0	0	84	119	330	0	84	12.0	360
0	0		125	600	0		11.5	520
0	0		119	330	0		10.9	340
0	0		127	340	0		10.9	420
0	0		112	770	0		12.0	450
0	0		122	910	0		11.9	710
0	0	74	117	800	0	74	12.9	740
120	3.0		128	1300	3.4		12.5	1240
120	5.6	102	145	1160	3.5	85	10.4	1020
120	2.9	99	13.3	900	-	86	10.5	800
120	3.8		15.4	710	1.5		11.5	590
120	4.2		16.5	740	1.6		12.4	680
120	3.6		18.0	910	1.8		11.7	750
120	1.0		18.7	700	1.5		13.1	680
120	2.9	88	17.4	740	1.4	76	12.5	590
0	2.1		16.7	530	1.5		11.8	490
0	0		15.9	690	0		12.3	470
0	0	58	14.4	710	0	68	13.3	650
0	1.2		13.5	940	1.1		11.8	930
0	0		14.4	990	0		11.9	940
0	0		14.3	700	0		12.4	600
0	0	72	12.7	650	0	76	12.2	590
0	0		11.9	580	0		11.4	500
0	0		12.0	610	0		12.5	520
0	0		11.9	740	0		11.2	550
0	0		11.9	710	0		12.3	560
0	0		11.4	720	0		12.0	660

Day of glucose tolerance test (see Figs 2 and 3)

intense depression of carbohydrate utilization occurred during the first three days of adrenocorticotrophic hormone administration. This was a period when nitrogen balance was being maintained. Unfortunately a glucose tolerance test was not done during this period. The period of negative nitrogen balance began on the fourth day of injections of adrenocorticotrophic hormone. Thus again we note the absence of a clear temporal correlation between nitrogen loss and the disturbance of carbohydrate metabolism. It is clear that in both subjects so far discussed (Subjects R S and A M) negative nitrogen balance and glycosuria were provoked by administration of adrenocorticotrophic hormone but it seems unlikely that either was dependent upon the other. Fig 2 shows that after seven days of injections a diabetic curve was obtained in Subject A M.

Subject M W showed the mildest effect of adrenocorticotrophic hormone upon carbohydrate metabolism of any of the three subjects studied (Table III and Fig 3) although she was treated on the same days and by the same procedures as those which apply to Subject A M. Variations among individuals and species differences in the response to adrenocorticotrophic hormone may account, in part, for the discordant results so far reported.

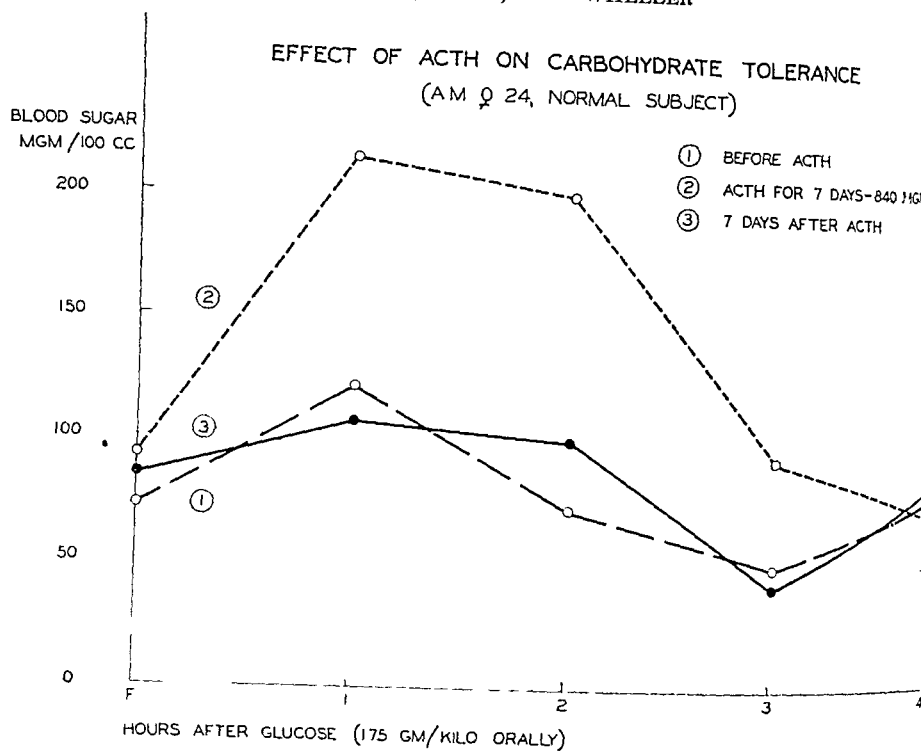


Fig 2

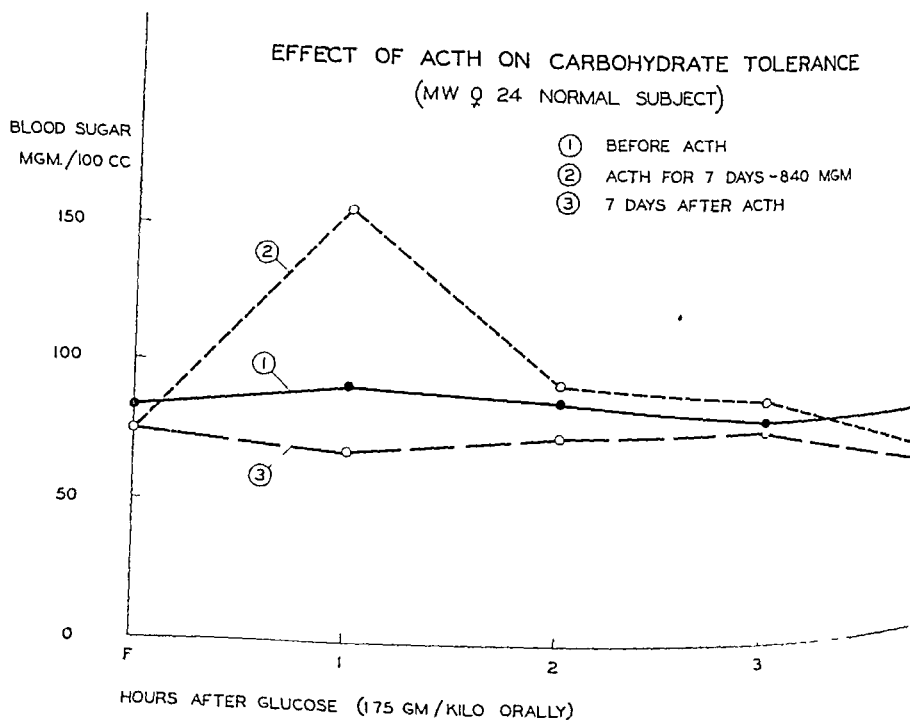


Fig 3



Table III shows that Subject M W exhibited glycosuria during the entire eight days of adrenocorticotrophic hormone administration. It is noteworthy that negative nitrogen balance was not produced in this subject at any time. In fact, the initial response was that of nitrogen retention. The sharp increase in urinary excretion of uric acid however was almost identical in absolute values, with that obtained for Subject A M. In all three subjects during the adrenocorticotrophic hormone periods the glycosuria shows a much closer correlation with the increased production of uric acid than with the state of nitrogen equilibrium.

Table III demonstrates another interesting finding. In both subjects glycosuria continued for one day after cessation of adrenocorticotrophic hormone administration and then ceased for forty eight hours. During this period urinary uric acid levels fell to the base line values. Fasting blood sugar determined on the morning of the third postinjection day was abnormally low for both subjects. On the following day glycosuria reappeared accompanied by a great increase in the urinary excretion of uric acid. These findings suggest that the following sequence of events occurred: (1) exogenous adrenocorticotrophic hormone depressed production of endogenous adrenocorticotrophic hormone; (2) cessation of exogenous adrenocorticotrophic hormone administration resulted in a short period of hypopituitarism with respect to adrenocorticotrophic hormone (in these subjects about forty eight hours); (3) this situation was at once reflected as hypoadrenocorticism (this interpretation is supported not only by the low levels of fasting blood sugar but also by the tremendous changes in electrolyte metabolism and in urinary steroid excretion which occurred during this same interval<sup>23</sup>), and (4) an intense stimulus finally evoked pituitary adrenal activity again. This rebound in pituitary activity from a dormant state releases a greater amount of corticoids than are needed and produces spontaneously the same metabolic changes in somewhat smaller degree that are observed on the first day of administration of exogenous adrenocorticotrophic hormone (Table III). It should be noted again that the spontaneous reappearance of glycosuria is related in time to the increased uric acid excretion rather than to changes in nitrogen metabolism.

#### SUMMARY

Three normal young adults were given 120 to 150 mg. of purified pituitary adrenocorticotrophic hormone intramuscularly daily in divided doses for eight to ten consecutive days. On the first day all subjects developed glycosuria which continued throughout the entire period of adrenocorticotrophic hormone administration. The glycosuria averaged 25.2, 3.8 and 2.5 Gm. per day for the respective subjects. Two of the subjects lost large amounts of body protein during the period of adrenocorticotrophic hormone injection despite an adequate protein intake. Significant negative nitrogen balance continued for five to six days following cessation of adrenocorticotrophic hormone administration but glycosuria ceased within twenty four hours of the last injection. Both of these subjects developed hyperglycemic plateau glucose tolerance curves during the adrenocorticotrophic hormone period. Normal carbohydrate tolerance

returned within one to two weeks after adrenocorticotrophic hormone was stopped

The third subject exhibited no loss of body protein. Nevertheless, glycosuria persisted during administration of adrenocorticotrophic hormone. Total glycosuria and loss of carbohydrate tolerance were less in this subject than in the other two.

Analysis of the data indicates that although greater loss of carbohydrate tolerance occurred in the two subjects who exhibited marked negative nitrogen balance, the glycosuria was not related either in time or in degree to the loss of body protein.

The data indicate, furthermore, that the loss of carbohydrate tolerance produced by adrenocorticotrophic hormone is the result of a depressed capacity of the tissues to utilize glucose (by oxidation and/or conversion to fat).

A close temporal correlation was observed in all three subjects between loss of carbohydrate tolerance and increased urinary excretion of uric acid during the periods of administration of adrenocorticotrophic hormone. Two subjects showed a spontaneous return of glycosuria on the fourth day after adrenocorticotrophic hormone had been stopped. In each case this was associated with a sharp increase of urinary uric acid. The temporary return of glycosuria and heightened uric acid excretion in the postadrenocorticotrophic hormone period was due to acute elaboration of endogenous adrenocorticotrophic hormone following a prolonged period of depressed productivity of endogenous adrenocorticotrophic hormone caused by administration of the exogenous hormone.

Observations made on one subject suggest the possibility that a heightened intracellular production of uric acid and its intermediaries produced by adrenocorticotrophic hormone, together with a fall of the levels of glutathione and perhaps other sulfhydryl-bearing substances, may diminish enzymatic production or release of pancreatic insulin. Such a mechanism could explain the close correlation between uric acid metabolism and carbohydrate metabolism which our data indicate.

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# A COMPARATIVE STUDY OF THE SERUM ALBUMIN GLOBULIN RATIO, THE CEPHALIN-CHOLESTEROL FLOCCULATION, AND THE THYMOL TURBIDITY TESTS FOR LIVER FUNCTION

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THE derangement in the formation of serum proteins which takes place in certain diseases of the liver may now be described quantitatively by determining the relative amounts of the protein fractions that are separated electrophoretically in the Tiselius apparatus<sup>1</sup>. An analysis of normal human serum having a total protein content of about 7.5 Gm per 100 ml shows 60 to 65 per cent in the albumin fraction and about 10 per cent in each of the  $\alpha$ ,  $\beta$ , and  $\gamma$  globulin fractions. In the serum of patients with liver disease the total protein content may be normal, but the proportions of the different fractions are usually changed. There is often a decrease in the albumin fraction and an increase in either or both of the  $\beta$  and  $\gamma$  globulin fractions. Heretofore the albumin globulin ratio of serum has been determined chemically by fractionation with high concentrations of salt to precipitate the globulins according to various methods,<sup>2</sup> and quite consistent results are possible when the procedures are carefully standardized. However, salt fractionation methods have fallen into some disrepute due to the difficulty of obtaining good duplicate values when all the factors involved are not properly controlled. Furthermore, recent studies have demonstrated that the values of the albumin-globulin ratio obtained by carefully fractionating the proteins of serum with salt do not reflect the actual partition of the electrophoretic patterns.<sup>3</sup> In 1945, Pillemer and Hutchinson reported a simple chemical method for the fractionation of the albumin and globulin of human serum by methyl alcohol in the cold which agrees quite well with the results of electrophoresis.<sup>4</sup> This method should prove useful in many laboratories since the Tiselius apparatus is elaborate and expensive and since the electrophoretic method at present does not seem to be adapted to general routine use.

Many tests for liver function have been devised which are based on the derangement in the formation of the serum proteins. Until recently these were only vaguely understood and their value was determined empirically from the agreement of the results with clinical findings. At present, two of these tests are widely used. The cephalin-cholesterol flocculation test of Hanger<sup>5</sup> measures the degree of precipitation twenty-four and forty-eight hours following the mixture of serum with an antigen emulsion prepared from sheep brain cephalin and cholesterol. The thymol turbidity test,<sup>6</sup> which was modified for quantitative photoelectric estimation by Shank and Hoagland,<sup>7</sup> measures the turbidity produced by adding serum to a buffered thymol solution. Many workers have

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compared the results of the two tests in liver disease<sup>8</sup> and it appears likely that they are not a measure of the same abnormality since in many instances the results do not agree. The mechanism of the cephalin cholesterol flocculation test has now been clarified by Moore and co workers with the aid of electrophoretic studies.<sup>9</sup> Two factors seem to be involved in a positive reaction. One is in the  $\gamma$  globulin fraction and the other is a reduction of the inhibition by albumin. The  $\gamma$  globulin fraction also appears to be involved in the colloidal gold reactions.<sup>10</sup> A positive reaction in the thymol turbidity test on the other hand seems to depend chiefly on the  $\beta$  globulin fraction and the lipids.<sup>11</sup>

Although the values for the partition of the proteins by electrophoresis have been compared with the results of liver function tests based on an alteration of serum protein, there is a paucity of data to compare the values of albumin and globulin determined by precipitation with methyl alcohol. In view of the simplicity of the latter method it seemed desirable to conduct a simultaneous study on a series of normal and pathologic sera to compare the results of the fractionation of the proteins by salt and by methyl alcohol with the cephalin cholesterol flocculation and the thymol turbidity tests.

#### EXPERIMENTAL

Unhemolysed serum was obtained from postabsorptive venous blood of hospital patients on the medical and surgical wards and from apparently healthy laboratory workers and nurses. The patients were divided into three groups. Group 1 consisted of ten patients with known liver disease. Group 2 represented eighteen cases in which changes in the serum proteins were probably due to nonhepatic causes such as nephrosis with albuminuria and hemorrhage. Group 3 consisted of seventeen patients with miscellaneous medical conditions in which no change in the serum proteins might be expected.

In most of the patients of Groups 1 and 2 at least two specimens of blood were analysed depending on the duration of hospitalization and on the clinical condition. One patient with acute hepatitis was followed throughout a period of two months in which there were abnormal findings. Although many routine laboratory determinations were made the present study is concerned primarily with the two chemical methods of determining the serum albumin and globulin and with the cephalin cholesterol flocculation and the thymol turbidity tests for liver function. Where it is pertinent the results of other determinations are included.

#### METHOD

The biuret reaction was used to determine the total serum proteins and the albumin in the filtrate after precipitating the globulins with either sodium sulfate or methyl alcohol. The same reagent, corresponding to reagent TP of Kingsley<sup>2b</sup> was used in each case and was prepared by adding 1,200 ml of 1 per cent crystalline copper sulfate to 6,000 ml of 14 per cent sodium hydroxide. The reagent was standardized frequently for total protein and for albumin with serum and with alcoholic or sodium sulfate filtrates of known protein nitrogen content determined by macro Kjeldahl for total nitrogen and by nesslerization for nonprotein nitrogen.

**Total Protein**—Total protein was determined in duplicate by adding 0.15 ml of serum to 10 ml of biuret reagent in colorimeter tubes. After fifteen minutes the solutions were read in an Evelyn photoelectric colorimeter at 540  $m\mu$ , with a blank tube containing 10 ml of the reagent and 0.15 ml of 0.9 per cent sodium chloride s.t for 100 per cent transmission. Some samples of serum were slightly turbid, but the solutions obtained on addition to the biuret reagent were clear except in one or two instances. About 2 ml of ether were added to every sample of turbid serum after mixing with the reagent and the solutions were then read in the colorimeter under the layer of ether. In every case the results were the same as those obtained in duplicate samples of the same serum without the use of ether. The protein content was calculated from a standard curve, which remained unchanged on repeated standardizations for several months. This curve was prepared from determinations of pooled serum of known protein nitrogen content which was diluted serially one to ten times with 0.9 per cent sodium chloride. The values of optical density,  $2 - \log$  of the transmission, were then plotted against concentration in per cent total protein. All the points fell on a straight line, but there was a slight deviation in the lower part of the curve, corresponding to a dilution of eight to ten times.

**Albumin by Salt Precipitation**—The method of Kingsley<sup>2b</sup> was used in somewhat modified form. The globulin was precipitated by adding 0.5 ml of serum to 9.5 ml of 9.4 per cent sodium sulfate contained in 15 ml centrifuge tubes. About 2 ml of ether were added and the tubes were stoppered and shaken vigorously several times. Within five minutes after mixing, the tubes were covered with rubber caps and centrifuged. A pipette was then inserted under the ether layer and the interphase precipitate, and 3 ml of the clear aqueous solutions were transferred to Evelyn tubes containing 10 ml of biuret reagent. A blank tube was prepared with 3 ml of a mixture of 9.5 ml of sodium sulfate solution and 0.5 ml of water added to 10 ml of the biuret reagent. The results were estimated from the reading of the colorimeter at 540  $m\mu$  on a standard curve prepared in the same way as for total protein, except that varying dilutions of a globulin filtrate of known protein content were used instead of whole serum. All the determinations were made in duplicate by precipitating separate samples of the same sera. Usually the results of the duplicate determinations agreed well within 0.1 Gm per 100 ml of serum, and the results were discarded if the agreement was less than 0.2.

**Albumin by Precipitation With Methyl Alcohol**—The method of precipitation was that of Pillemer and Hutchinson.<sup>4</sup> The serum and reagents were placed in a bath of ice water, where they were mixed, and the precipitated globulin was filtered in the refrigerator. Albumin was determined by adding 1 ml of the cold filtrate to 10 ml of the biuret reagent and reading the resultant color in the Evelyn colorimeter at 540 millimicrons. The results were calculated from a standard curve prepared from the values of ten diluted samples of an alcoholic globulin filtrate of known protein nitrogen, determined by macro Kjeldahl which was corrected for nonprotein nitrogen. The accuracy of the method was checked with a series of thirty determinations precipitated in duplicate which, for the most part, agreed within 0.1 Gm of albumin per 100 ml of serum. All the other determinations were made on duplicate samples of the globulin filtrates.

**Thymol Turbidity Test**—The method of Shank and Hoagland<sup>7</sup> was used in an essentially unmodified form. The reagent was prepared and standardized with mixtures of 0.09 per cent barium chloride and 0.200N sulfuric acid, using an Evelyn colorimeter with 660 filter. For the tests, 0.15 ml of serum was added to 10 ml of the reagent, and the readings were compared against a blank tube containing only the reagent, which was set for 100 per cent transmission.

**Cephalin Cholesterol Flocculation Test**—The test was made with commercial antigen<sup>8</sup> according to the method of Hanger<sup>5</sup> by adding 1 ml of the antigen emulsion to a mixture of 0.2 ml of serum and 4 ml of 0.85 per cent sodium chloride in 15 ml centrifuge tube. After mixing, the tubes were stoppered and placed in a dark closet at room temperature, and the amount of flocculation in the tubes was estimated, without centrifugation, after twenty-four and forty-eight hours. Since it was difficult to estimate differences in the amount of

\*Obtained from Difco Laboratories, Detroit, Mich.

precipitate by measurement in graduated tubes the supernatant fluid above the sedimented precipitates was compared with standards which were prepared at the time of the readings. The standards consisted of serial dilutions of a mixture of 2 ml of the antigen emulsion and 84 ml of 0.85 per cent sodium chloride corresponding to the dilution of the antigen with serum. The following criteria were used to evaluate the results 1 plus is intermediate between the undiluted standard and a dilution of twofold, 2 plus is twofold 3 plus is four to eightfold, 4 plus is water clear

## RESULTS AND DISCUSSION

It is seen in Table I that the albumin globulin ratio in eleven normal healthy individuals varied between 1.46 and 1.92 by the method of precipitation with methyl alcohol. These values compare favorably with those obtained in normal subjects by the electrophoretic method<sup>1</sup>. The albumin globulin ratios determined by fractionation with salt are higher in almost every case but this is consistent with recent findings<sup>1</sup> that the  $\alpha$  globulin fraction remains in solution in about 21 per cent sodium sulfate. As was to be expected in normal individuals, the thymol turbidity and the cephalin cholesterol flocculation tests yielded normal values.

TABLE I COMPARATIVE VALUES IN NORMAL SUBJECTS

SEX	AGE (YE)	TOTAL PROTEINS (GM PER 100 ML)	ALBUMIN/GLOBULIN RATIO		THYMOL TURBIDITY (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION	
			SALT	ALCOHOL		24 HR	48 HR
F	21	7.5	1.76	1.72	0	1 +	2 +
F	30	7.24	2.42	1.67	0.9	1 +	1 +
F	25	6.94	2.24	1.92	0	±	1 +
F	31	6.54	2.06	1.77	0	2 +	2 +
F	25	7.56	2.24	1.56	0	±	1 +
F	30	7.38	1.82	1.50	0	1 +	2 +
F	30	7.49	1.84	1.64	0	1 +	2 +
F	25	8.15	1.80	1.59	0.9	—	2 +
F	21	7.44	2.15	1.51	1.5	—	2 +
M	35	6.74	2.04	1.68	0	1 +	1 +
M	35	8.05	2.02	1.46	1.4	±	1 +

In Table II it is seen that the values of the albumin globulin ratio determined by fractionation with methyl alcohol follow the clinical course in liver disease very well and the values of the ratio determined by salt fractionation appear only slightly less consistent. In the cases of parenchymatous liver disease which are presented the values of thymol turbidity also seem to afford a reliable guide but the cephalin cholesterol flocculation test in our experience served only to indicate the period in which the disease was at its height.

In Patient H. S. there was a slight clinical improvement early in the disease, although both the albumin globulin ratio and the thymol turbidity test remained essentially constant. About five weeks after admission during a relapse in the clinical condition of this patient the cephalin cholesterol flocculation test became positive. Thereafter with the onset of steady clinical improvement the values of both the protein ratio and the thymol turbidity test gradually returned to normal. At biopsy on June 12 the liver showed evidence of almost complete healing. From the values of the albumin globulin ratio it would seem that the hepatic function had returned to normal.

TABLE II COMPARATIVE VALUES IN CASES OF LIVER DISEASE

PATIENT	DATE	TOTAL PRO TEIN (PER CENT)	ALBUMIN GLOBULIN RATIO		THYMOL TUR BIDITY (UNITS)	CEPH CHOL FLOCCULATION		REMARKS
			SALT	MICROHOL		24 HR	48 HR	
a Acute hepatitis								
H S	4/29	6.91	0.93	0.80	7.6	±	1	
	5/16	6.58	0.61	0.80	12.9	2	2	
	5/19	6.63	53	0.82	8.7	2	2	Clinical improvement
	6/3	8.02	86	93	17.7	4	4	Cholesterol esters 49%
	6/6	8.58	85	—	20.6	3	4	Relapse
	6/18	7.15	1.27	1.14	10.7	2	2	
	6/19	7.13	1.26	1.17	9.6	2	2	Clinical improvement
	6/30	7.30	1.54	1.48	4.7	2	2	Cholesterol esters 49%
M L	7/10	7.05	1.74	1.40	5.0	1	1	
	7/12 Biopsy subsiding hepatitis							
	6/16	6.60	1.13	0.60	17.8	3	3	
B R	6/25	6.98	0.96	0.80	14.5	—	3	
	7/8	6.88	1.01	0.95	11.1	2	2	
	6/11 Biopsy diffuse hepatitis							
B R	7/2	7.68	1.22	0.67	14.2	—	2	
	7/21	7.72	1.08	1.06	7.0	2	2	
	9/25	7.60	—	1.38	7.7	1	1	Complete clinical recovery
b Cirrhosis with liver failure								
B C	6/12	6.98	0.29	0.91	10.4	3	3	
	6/17	7.03	0.26	0.94	12.6	3	4	Clinical improvement
	6/30	7.10	0.32	1.16	8.4	4	4	
	7/28	7.53	—	1.24	13.1	3	3	
c Common duct obstruction								
B D	5/15	8.10	0.90	1.02	1.9	2	2	
P P	6/3	7.25	1.68	1.32	1.8	0	0	
S S	5/27	8.12	1.15	1.26	1.0	0	±	
F K	6/4	6.52	1.48	0.55	0	0	±	
d Metastatic carcinoma of the liver								
R S	5/14	6.74	0.97	0.71	0.3	0	0	
I T	5/2	7.54	0.98	0.80	2.0	±	1	

In Patient M L a biopsy on June 11 showed extensive liver damage. This was in agreement with the low ratio of the proteins and the high values of the thymol turbidity. In the case of Patient B R, who showed a typical clinical picture of acute hepatitis the values of the ratio and the thymol turbidity were also consistent. Patient B C had a typical case of cirrhosis of the liver. He was admitted to the hospital gravely ill, but steadily improved under treatment and was finally discharged. Several weeks later the patient was sufficiently well to return for the last examination. In Table II it is seen that the values of the albumin-globulin ratio were quite consistent with the steady improvement shown clinically, although the thymol turbidity and cephalin-cholesterol flocculation tests remained elevated and essentially constant.

In obstruction of the common duct and in diffuse carcinoma of the liver the values of the albumin-globulin ratio determined with methyl alcohol are consistently depressed. This depression was especially of value in the case of Patient I T (Table II), whose liver was riddled by metastatic carcinoma of renal origin and who died very shortly after laparotomy from hepatic and renal failure. Yet in his case as well as in the other case of extensive carcinoma, and



in the cases of relatively long standing obstructive jaundice the thymol turbidity and cephalin cholesterol flocculation tests gave normal values and afforded no index to the marked hepatic damage present

In Table III are presented the results of all the nonhepatic cases studied in which a derangement of the serum proteins might be expected. The patients of this group had conditions entailing a loss of protein either in the urine or as a result of hemorrhage. Edema was common. It is seen that the albumin globulin ratio determined by methyl alcohol precipitation was approximately

TABLE III COMPARATIVE VALUES IN CASES SHOWING A DERANGEMENT OF SERUM PROTEINS DUE TO NONHEPATIC CAUSES

PATIENT	DATE	TOTAL PROTEIN (%)	ALBUMIN/GLOBULIN RATIO		THYMOL TURBIDITY (UNITS)	CEPHALIN CHOLESTEROL FLOCCULATION	
			SALT	ALCOHOL		24 HR.	48 HR.
J C	6/19	3.94	1.16	1.03	0	1	1
	7/8	5.71	1.14	0.55	2.1	1	1
		<i>Bleeding duodenal ulcer</i>					
E B	5/1	6.19	1.22	0.64	1.1	±	±
		<i>Carcinoma of stomach with peripheral edema</i>					
S G	5/20	7.05	0.68	0.97	0	0	0
		<i>Toxemia of pregnancy with edema</i>					
I G	5/2	5.46	1.48	0.81	1.5	0	±
		<i>Hypertensive heart disease with edema</i>					
J H	4/18	5.49	1.04	0.49	0.5	0	0
	5/12	6.44	1.06	0.82	0	0	0
	6/5	5.97	0.97	0.71	—	—	—
		<i>Severe burns</i>					
A C	5/14	7.04	1.02	0.95	3.7	2	3
		<i>Profuse vaginal bleeding</i>					
M K	5/21	6.41	0.83	1.05	2.4	2	2
	5/22	6.66	0.83	1.12	4.1	1	1
		<i>Carcinoma of prostate with marked anorexia</i>					
J K	5/22	5.32	1.26	1.14	0	±	±
	5/26	5.66	1.32	1.06	0	±	±
		<i>Bleeding gastric ulcer gastrectomy</i>					
B I	5/20	6.05	0.72	0.67	0	0	0
		<i>Multiple myeloma</i>					
J C	5/7	5.62	1.85	0.59	2.7	0	0
		<i>Acute gangrenous appendicitis</i>					
F K	6/9	6.03	1.54	0.64	2.0	0	0
	6/12	5.8	1.41	0.81	0.4	0	0
		<i>Fracture of neck of femur peripheral edema</i>					
E K	5/8	8.85	0.65	1.44	10.6	3	2
		<i>Boeck's sarcoidosis</i>					
K I	4/23	7.70	1.47	1.60	14.4	±	1
		<i>Chronic lymphadenitis</i>					
B F	4/24	6.90	1.67	0.94	0.1	0	0
		<i>Chronic pyonephritis</i>					
F M	4/22	6.24	1.28	0.76	2.1	0	0
	5/5	6.81	1.51	1.57	1.9	0	0
		<i>Acute nephritis with edema</i>					
B S	4/28	4.20	1.19	0.60	2.7	0	0
	5/5	4.1	0.61	0.20	3.3	0	0
	5/19	4.07	0.66	0.19	1.9	±	±
	5/27	4.32	0.46	0.14	7.9	0	0
		<i>Nephrosis with severe albuminuria and edema</i>					
L S	4/30	5.81	1.24	0.90	1.7	±	±
		<i>Acute glomerular nephritis</i>					
I G	5/26	7.23	1.63	1.14	1.9	±	±
		<i>Nephrosis</i>					

normal in only two of the seventeen patients. The clinical diagnoses in these cases were Boeck's sarcoidosis and chronic lymphadenitis. It is noteworthy that the thymol turbidity test was elevated in both. Another patient, Patient B L who was diagnosed as having multiple myeloma, had a markedly depressed ratio, but both the thymol turbidity and the cephalin-cholesterol flocculation tests were normal. The increased  $\beta$  globulin fraction which other workers find in this disease<sup>13</sup> did not yield elevated values in the thymol turbidity test.

TABLE IV COMPARATIVE VALUES IN MISCELLANEOUS HOSPITAL CASES NOT USUALLY ASSOCIATED WITH CHANGES IN SERUM PROTEINS

PATIENT	TOTAL PROTEIN (%)	ALBUMIN/GLOBULIN RATIO		THYMOL TURBIDITY (UNITS)	CEPH CHOL FLOCCULATION		DIAGNOSIS
		SALT	ALCOHOL		24 HR	48 HR	
1	6.92	1.58	2.20	7.2	±	1	Chronic nephritis
2	7.42	1.90	1.54	11.4	±	±	Colostomy
3	6.31	1.80	1.58	1.4	±	1	Chronic cholecystitis
4	6.61	1.33	1.83	1.8	0	±	Nutritional anemia
5	6.75	1.43	0.85	4.5	0	1	Prostatic hypertrophy
6	7.26	1.46	2.26	2.8	±	±	Chronic nephritis
7	8.55	0.92	1.46	10.9	±	±	Essential hypertension
8	7.02	1.24	1.16	6.2	1	2	Chronic rheumatoid arthritis
9	6.18	1.02	1.71	0.7	2	3	Chronic cholecystitis
10	7.50	1.40	1.96	5.1	3	3	Duodenitis
11	7.05	1.54	1.92	0.6	±	±	Essential hypertension
12	6.51	1.58	1.53	0.1	±	±	Pneumonia
13	6.85	1.78	1.48	1.3	0	0	Cerebral accident
14	7.10	1.00	1.78	0	±	±	Cardiac disease
15	5.84	1.82	1.24	0.7	1	1	Pernicious anemia
16	8.28	1.01	1.39	4.0	2	3	Tuberculous lymphadenitis
17	6.57	1.71	1.46	0.8	1	1	Chronic lymphatic leucemia

In Table IV are seen the results in an unselected group of miscellaneous hospital patients in whom there was no suspicion of liver disease or of any other pathology that might cause a derangement in the serum proteins. Only three of the seventeen patients showed a marked depression of the albumin globulin ratio. Of the seventeen patients, four showed an elevated thymol turbidity and three a positive cephalin-cholesterol flocculation.

It seems clear that the albumin-globulin ratios determined by precipitation with methyl alcohol more accurately reflect the severity of the pathology in patients with liver disease than any other individual test and may be used as an index of the extent to which the liver is involved. In Tables III and IV it is seen that the abnormalities in the serum proteins due to nonhepatic causes can be correlated quite well with the clinical picture, but the values obtained by salt fractionation appear to be less consistent than those obtained by precipitation with methyl alcohol. In our experience the results of the latter method are even more reliable than the values of the thymol turbidity test provided

we rule out clinically, as far as possible, nonhepatic causes for a derangement in the serum proteins. Particular emphasis is placed on the fact that Table IV contains the results in all the cases studied where a derangement was not expected clinically.

Whereas Shank and Hoagland found the values of the thymol turbidity test in forty six normal subjects to range between 0 and 4.7 units, our range for a smaller series of normal healthy individuals is 0 to 1.5 units. One value of 9.5 units was discarded, since it was found in a man who was known to use alcohol to excess and in whom the albumin globulin ratio by methyl alcohol precipitation was 0.92. The cephalin cholesterol flocculation test in this individual was 2 plus. The latter test in our series of eleven normal subjects showed seven with a value of 2 plus and four with a value of 1 plus. A reading of 3 plus or 4 plus is generally considered abnormal for the Hanger test. Since control tubes without added serum were always 1 plus or less, this high proportion of apparently healthy individuals with values of 2 plus is disturbing. We have no explanation for the discrepancy between our results and those of other workers who almost invariably obtained negative values with commercial antigen in normal subjects.<sup>8</sup> However, in spite of this discrepancy our results with the cephalin cholesterol flocculation test seem quite consistent and apparently indicate the height of the disease in parenchymatous liver involvement. We stress the fact that our procedure entails an objective comparison of the unknown sera with standard tubes prepared from the antigen and saline solutions.

Of interest in connection with the serum proteins in liver dysfunction is the high value of the thymol turbidity test found in Patient B. S. (Table III) a few days before death. The total proteins in this patient had remained constant at about 4 Gm. per 100 ml. for a month, and there was a constant elimination in the urine of about 15 Gm. of protein per day. Transfusion was not resorted to, but the patient was kept on a high protein diet which was apparently sufficient to replace the albumin that was excreted. Death finally came as a result of an abdominal infection which may have involved the liver. Permission for an autopsy was not obtained. Although the absolute amounts of albumin and globulin were essentially constant in three different determinations over a period of three weeks, the thymol turbidity test was positive only in the last determination with a value of 7.9 units.

In conclusion the present study demonstrates the importance of the albumin globulin ratio in the study of liver disease. Neither the thymol turbidity nor the cephalin cholesterol flocculation tests indicated an involvement of the liver in the cases of obstruction of the common duct and of carcinoma of the liver which were studied, but all showed a marked depression of the ratio. There is good reason to believe that the values of the proteins obtained by precipitation with methyl alcohol are a true estimate of the partition that is shown by electrophoresis. Although it is obviously not possible in every case to be certain that nonhepatic causes for a derangement of the serum proteins are not present, our results appear encouraging. We feel that once hepatic involvement is recognized, the albumin globulin ratio becomes a valuable means of

evaluating the degree of the involvement. It is also of aid in gauging the clinical progression or regression of the cases under study, and this can be of prognostic significance.

#### SUMMARY

The results of a simultaneous study of the albumin globulin ratio, the thymol turbidity test, and the cephalin-cholesterol flocculation test in eleven normal subjects and in three groups of hospital patients are presented. The albumin-globulin ratio, as determined by fractionation with methyl alcohol, was found to be consistently depressed and to agree with the general clinical picture in ten cases of liver disease. The thymol turbidity test also was found to give consistent results in four cases of parenchymatous liver disease, but offered no index to the dysfunction in two cases of liver carcinoma and in four cases of relatively long-standing obstructive jaundice. The cephalin-cholesterol flocculation test seemed only to indicate the height of the disease in the four cases of parenchymatous liver disease which were studied.

That nonhepatic causes for a decrease in the albumin globulin ratio can be excluded with a fair degree of certainty is shown by the low incidence of a depressed ratio found in a group of miscellaneous hospital cases which did not include patients in whom a low ratio might be expected from causes entailing a loss of serum protein. It is concluded that the albumin globulin ratio may be utilized with profit in the study of liver disease, since it seems to offer information which is as good as either the thymol turbidity or the cephalin-cholesterol flocculation tests.

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# A DILUTION TURBIDITY TEST IN THE SERUM IN COMPARISON WITH THE THYMOL TURBIDITY AND CEPHALIN CHOLESTEROL FLOCCULATION TESTS

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SINCE the detection of hyperglobulinemia is of importance, a large variety of methods has been employed to discover an increase, either absolute or relative, in plasma globulins. Whereas previous work has tried to link the results of the tests for hyperglobulinemia with the amount and distribution of protein determined by chemical methods (salting-out techniques<sup>1</sup>), more recent studies have attempted to correlate some of these well established clinical tests with definite serum protein electrophoresis. It soon became evident that rather complicated mechanisms are involved and not simply an increase of a single, well-defined fraction of the serum protein. An inquiry into the nature of Hanger's cephalin-cholesterol flocculation test<sup>2</sup> (C C F), for example in infectious hepatitis, has led to the conclusion that the gamma globulin content of the serum has to be increased and that its albumin content as well as the protective action of this albumin has to be decreased in order to give a positive result in this test<sup>3</sup>. The substance responsible for the turbidity in MacLagan's thymol turbidity test<sup>4, 5</sup> (T T T) was originally assumed by MacLagan himself to be composed of gamma globulin, phospholipids, cholesterol, and thymol, whereas Cohen and Thompson<sup>6</sup> reported that the thymol reagent reacts chiefly with the beta globulin fraction of the serum. In addition, Recant, Chargaff, and Hanger<sup>7</sup> have demonstrated that the mechanisms of the two tests are different. They found that the gamma globulin fraction is not necessary for a positive thymol test but is essential for a positive cephalin cholesterol flocculation and that the presence of lipids is essential in the thymol turbidity test but has no influence in the cephalin-cholesterol flocculation test. According to these workers, the albumin fraction, on the other hand, is of importance for the cephalin-cholesterol flocculation but not for the thymol turbidity test. Cohen and Thompson, it is true, have also stated that there is not only no correlation between a gamma globulin increase and the thymol turbidity test units, but also that the same holds true for the beta globulin in their experiments. They therefore consider a change in the nature or amount of lipids bound to the beta globulin fraction as a possible agent. Kunkel and Hoagland<sup>8</sup> in a very elaborate communication have recently presented evidence that the turbidity measured in the thymol turbidity test depends on protein as well as on lipids precipitated. The varying lipid content of sera, however, determines different degrees of turbidity only in the presence of serum globulin likely to produce the reaction. Their electrophoretic analysis of the thymol precipitate after lipid removal demonstrated the protein to be a gamma globulin, study of the sera after separation from the

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thymol precipitate, in contrast showed a definite decrease in beta globulin. The addition of large amounts of albumin *in vitro* proved to have a slight inhibitory effect on the thymol precipitation. The authors conclude that the thymol turbidity in infectious hepatitis depends on the presence of lipids and of abnormal lipid protein complexes migrating in the beta globulin fraction of the serum. The gamma globulin fraction of serum also plays an important role in the reaction."

Kala azai has long been the yardstick by which the efficacy of methods for detecting hyperglobulinemia was measured. Out of the necessity for a simple screening test for this disease a blood or serum dilution test was devised by Brahmachari<sup>9</sup> who used a 1:2 to 3 dilution with distilled water. Ray<sup>11</sup> in 1921 and Sia<sup>12</sup> in 1924 suggested a 1:200 or 1:30 dilution respectively using whole blood. It is of interest to mention that Naumann<sup>13</sup> showed that water saturated with alveolar air (40 mm Hg CO<sub>2</sub>) precipitates certain globulins from the serum.

Our interest in this subject was aroused by the recent determination of this dilution fraction and of cold precipitable globulins by Wertheimer and Stein in kala azar<sup>14, 15</sup> and by Wertheimer<sup>16</sup> in other hyperglobulinemic states. In the course of an investigation into this cold fraction it seemed worth while to re-evaluate the procedure of serum water dilution. Because of the uncertainty concerning the nature of the material precipitated and because of the findings that became evident during this work, we prefer to call the test as performed here a dilution turbidity test (D.T.T.). Since it was intended to find out whether this reaction is indicative of some distinct hyperglobulinemic pattern the thymol turbidity and the cephalin cholesterol flocculation tests were carried out at the same time. They may serve as possible range finders for the elucidation of this hypothetical pattern (roughly beta or gamma globulin lipid dependence, and so forth).

#### MATERIAL AND METHODS

Sera of patients from various departments of this hospital were examined.

The dilution test was performed in the following way: 0.4 cc of fresh serum were diluted into 60 cc of doubly distilled water (1:15 dilution) and gently mixed. The resulting turbidity was observed after a few minutes and its intensity was measured in a Fisher photometer (filter 660 as used for the thymol turbidity determination) after thirty minutes. The readings so obtained were then converted into units using the barium sulfate standards of Shank and Hoagland<sup>1</sup> just as for the determination of units in the thymol turbidity test. It soon became evident, first that any value exceeding 20 units represented an increase in this fraction and consequently an abnormal result and second that a twenty-four hour flocculation could frequently be observed. This flocculation appeared in many of the sera which showed an abnormal increase in turbidity.

The eye quickly becomes used to gauging low normal and high values even without the use of a photometer.

The serum so diluted loses its color entirely except in the presence of a fair increase in bilirubin, in contrast to the thymol turbidity and cephalin-cholesterol flocculation tests.

The thymol turbidity test was carried out according to MacLagan's original technique with Shank and Hoagland's modification and using the suggestion of MacLagan<sup>4</sup> and of Neefe and Reinhold<sup>18</sup> that the flocculation which takes place after twenty-four hours in certain sera be noted. Values over 4 thymol turbidity units as well as flocculations of 1 plus to 4 plus were considered abnormal.

The cephalin cholesterol flocculation test was performed according to Hanger's original method.<sup>2</sup> The test tubes for all three tests were kept in a darkened room as suggested by Neefe and Reinhold<sup>18</sup> for the cephalin cholesterol flocculation test. A plus minus reading was considered negative.

Since this hospital cares mostly for the chronically sick, the material examined reflects this fact in the relatively low number of certain acute conditions commonly examined in a study of this sort. This circumstance, on the other hand, provided us with a chance to become well acquainted with the so called false positives encountered in performing these tests.

#### EXPERIMENTAL RESULTS

Early in the course of this investigation it became evident that the dilution turbidity paralleled the thymol turbidity rather closely. However, a certain number of discrepancies occurred. These discrepancies could be divided into two principal groups: first, those apparently due to a real difference in the two fractions of the serum responsible for the two respective reactions, and second, others which proved to be dependent on a time factor in the carrying out of the dilution, both will be discussed later.

It seemed, therefore, of interest to perform several experiments designed to elucidate the relationship between the dilution turbidity and the thymol turbidity on the cephalin cholesterol flocculation and their respective biochemical backgrounds. By and large, procedures carried out by Recant and co-workers<sup>7</sup> were applied with certain modifications.

The thymol turbidity test and the dilution turbidity test are both performed using distilled water as a diluent, whereas the cephalin cholesterol flocculation is done in normal saline solution. Both the former tests are read after thirty minutes. A series of successive dilutions of normal saline solution demonstrated (Table I) that the dilution turbidity increases with decreasing salt concentration, pointing to its euglobulin nature.

TABLE I INFLUENCE OF SODIUM CHLORIDE CONCENTRATION ON THE DILUTION TURBIDITY

SERUM	0.4 ML SERUM IN 6 ML H <sub>2</sub> O	0.23% NaCl	0.45% NaCl	0.9% NaCl
B. S.	5	2	1	0.5
I. R.	6		1	1
D. R.	2.5		2	1.5

According to McFarlane's technique<sup>19</sup> (repeated lipid extraction with ether and separation of extract by freezing), extracts of several sera were prepared. The thymol turbidity, dilution turbidity, and cephalin cholesterol flocculation of these sera were determined immediately before and after the extraction. As Table II shows, this procedure was followed by a considerable decrease in thymol turbidity and dilution turbidity and, in contrast to the findings of Recant and co-workers,<sup>7</sup> also by a slight but noticeable decrease in cephalin cholesterol flocculation of about 1 plus. This decrease in cephalin cholesterol flocculation may be due to an alteration of the protein structure caused by the repeated freezings, rather than to the extraction of lipid material.

The dried substance extracted by the freezing process in seven sera did not dissolve in KOH, and it gave a negative biuret reaction in five and a very mildly positive reaction in two of the extracts. The positive reactions may



TABLE II RESULTS OF DILUTION TURBIDITY THYMOL TURBIDITY AND CEPHALIN CHOLESTEROL FLOCCULATION TESTS ON SEVEN SERA BEFORE AND AFTER LIPID EXTRACTION BY FREEZING WITH ETHER (TECHNIQUE OF McFARLANE<sup>19</sup>)

SERA	DIAGNOSIS	DATE (1947)	DTT		TTT		CCF	
			BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER
G D	Cirrhosis of liver	9/2	7 3+	1	11 3+	2	4+	3+
T Z	Infectious hepatitis	9 -	5 +	1	12 3+	2	3 plus	3plus
E S	Arteriosclerotic heart dis ease, congestive failure	9/9	3	0.5	3.5	1.5	Neg	Neg
M B	Rheumatic heart disease, congestive failure	9/9	2.5	1	2	1.5	Neg	Neg
J W	Pneumoconiosis	9/9	2.5	1	7.5	1.5	Neg	Neg
N P	Cirrhosis and carcinoma of liver	9/9	2	0.5	6 +	1.5	plus	1 plus
M L	Cirrhosis of liver	9/9	12.5 4+	5 3+	20 4+	5 2+	4 plus	3 plus

+ Slight flocculation -+ moderate flocculation 3+ heavy flocculation 4+ complete flocculation

have been due to a slight contamination of the extract with serum. The extracted substance is apparently a lipid fraction of the serum. It was interesting to note that the extracts of sera having high thymol turbidity and dilution turbidity values prior to extraction yielded a visibly greater amount of substance.

TABLE III RESULTS OF DILUTION TURBIDITY, THYMOL TURBIDITY AND CEPHALIN CHOLESTEROL FLOCCULATION TESTS BEFORE AND AFTER ADDITION OF SERUM LIPID EXTRACTS OBTAINED FROM OTHER SERA

SERA	DTT	TTT	CCF	PLUS EXTRACT OF SERA	DTT	TTT	CCF
D R	-	2.5	-	T Z	13	12	-
E S	2.5	3.5	Neg	M I	9	9	Neg
					+	+	
N P	2	8.0	2 plus	M B	10.5	11	Neg
					+	+	

- Not examined

Table III shows the effect of adding these extracts on a quantitatively corresponding basis to other sera and the subsequent behavior in tests. To an amount of 10 cc of serum the extract obtained from 10 cc of another serum was added and the serum so enriched was used for the determinations. The extracts

TABLE IV RESULTS OF DILUTION TURBIDITY, THYMOL TURBIDITY AND CEPHALIN CHOLESTEROL FLOCCULATION TESTS CARRIED OUT ON SERUM LIPID EXTRACTS

EXTRACTS OF SERUM	DTT	TTT	CCF
T Z	10.5	7	-
M B	6	5.5	Neg
M L	6.5	6.5	Neg
	+		

- Not examined

themselves gave positive thymol turbidity and dilution turbidity tests but negative cephalin cholesterol flocculation tests (Table IV).

Similarly the following two analyses bear out the conformity of behavior of dilution turbidity and thymol turbidity in contrast to cephalin cholesterol flocculation.

The dilution turbidities in the blood sera of three healthy rabbits were 10, 10, 10, and the thymol turbidities were 10, 10, and 0.5, respectively, the cephalin-cholesterol flocculations were 4 plus, the two latter being in agreement with the findings of Recant, Chaigaff and Hanger.<sup>7</sup>

Human immune serum globulin\* was tested. Table V demonstrates the results, proving that this product contains a large amount of cephalin cholesterol flocculation producing substance (gamma globulin) and apparently very little of the materials responsible for the two other tests.

TABLE V RESULTS OF DILUTION TURBIDITY, THYMOL TURBIDITY, AND CEPHALIN CHOLESTEROL FLOCCULATION TESTS ON HUMAN IMMUNE SERUM GLOBULIN (SQUIBB)

AMOUNT OF MATERIAL USED (ML)	D T T	T T T	C C F
0.4	1		
0.2	0.5	1	4 plus*
0.1	0.5		4 plus*
0.05			4 plus*
0.025			4 plus†
0.01			4 plus†
0.005			4 plus†
0.0025			4 plus†

\*Flocculation starting after five minutes

†Flocculation starting after about thirty minutes

We feel justified in concluding from the foregoing experimental and biologic findings that the serum fraction responsible for a positive dilution turbidity test is closely related to the one responsible for a positive thymol turbidity test.

At the beginning of our work, a fair proportion of discrepancies between dilution turbidity and thymol turbidity (the dilution turbidity was too low compared with the thymol turbidity) was found to be due to a delay in performing the tests. The later a dilution turbidity test was carried out, the lower its value was likely to be, there was, however, some turbidity even after five to six hours. Moreover the increased dilution turbidity of pathologic sera showed a definite tendency to persist longer.

Since there was reason to suspect that loss of CO<sub>2</sub> might account for this inconstancy of findings, a series of experiments was performed in order to study this possibility. In these experiments samples of blood were collected under oil, the serum was separated under oil in some of them, and the tests were performed on the sera thus obtained. These results were compared with those obtained on blood collected in the usual way. Table VI summarizes a few of these experiments. The results led to the following conclusions: dilution turbidity tests done on separated sera remain more constant than those done on unseparated sera, dilution turbidity obtained from sera or even from unseparated sera under oil tends to keep constant for a longer time (about as long as the protective action of oil on the CO<sub>2</sub> content can be expected to last, several hours according to Peters and Van Slyke<sup>20</sup>). However, apparently there are other factors which must have accounted for the rather erratic behavior of the dilution turbidity after six hours or more. With the intention of excluding at

TABLE VI CHANGES IN DILUTION TURBIDITY VALUES UNDER OIL AND WITH SERUM SEPARATED FROM CLOT

SERA	HOURS	CLOT PLUS SERUM	SERUM SEPARATED	CLOT PLUS SERUM UNDER OIL	SERUM SEPARATED UNDER OIL
F	Initial value	15		25	
	2½		2	25	25
	4		15	25	25
	5½		10	25	25
	24			3	
B	Initial value	6		6	
	1½		5	6	6
	4		5	6	6
	24	5	25	6	6
D	Initial value	7		7	
	1	5		7	
	3	5		8	
T Z	Initial value	7		7	
	1	6		7	
	18	5		7	

least one other possibility an attempt was made to poison the active enzyme systems which may be responsible for the deterioration of the dilution turbidity. The addition of 0.05 cc of a 1:10,000 solution of NaCN to 1.0 cc of serum however did not prevent the decrease as observed in the controls.

When the dilution turbidity test is performed in distilled water saturated with CO<sub>2</sub> (according to Naumann<sup>13</sup>) but otherwise following the technique employed throughout our work the values thus obtained are higher than when the test is performed on freshly drawn blood using distilled water alone. The normal upper limit here is probably close to 4 units.

We consequently consider it as probable that the escape of CO<sub>2</sub> is the reason for the gradual lowering of the dilution turbidity values on standing. To avoid using means for controlling this escape and thereby complicating an otherwise simple procedure we recommend that the test be performed shortly after the blood is drawn preferably within one to two hours. Otherwise the serum should be separated from a blood specimen taken under oil and stored under oil in a refrigerator. Even taking these precautions it is best not to delay the test for more than six hours.

In the following tables (Tables VII to X) and statistics only those values are used which were obtained from freshly examined specimens.

#### CLINICAL RESULTS

In 328 blood specimens taken from 203 patients the following results were obtained. 196 specimens from 105 patients showed a positive result in one or more of the tests performed. The dilution turbidity test was positive in 139, the thymol turbidity test in 160 and the cephalin cholesterol flocculation in 131. All three tests were positive simultaneously in 90 specimens. Only the dilution turbidity and thymol turbidity tests were positive in 34. The dilution turbidity test and cephalin cholesterol flocculation test were positive in 6. The thymol turbidity and cephalin cholesterol flocculation tests were positive together in 14. The dilution turbidity test was positive alone in 9, the thymol turbidity test alone in 22 and the cephalin cholesterol flocculation alone in 21.

TABLE VII DISEASES OF THE LIVER AND BILIARY TRACT

PATIENT	DIAGNOSIS	DATE (1947)	D T T	T T T	C C F	OTHER FINDINGS
E R	Infectious hepatitis (Improving)	6/18 7/2 7/7 7/16 7/28	6 4 3 5 4 2 5	11 7 5 6 8 5	4 plus 3 plus ± Neg Neg	Bilirubin 12 7 mg % dir 9 7 0 8 1 8
L G	Infectious hepatitis, HCVD <sup>†</sup> (Improved) (Improved) (Well)	6/23 6/27 7/7 8/11	3 5 4 3 5 4 2 5	6 6 6 6 2	4 plus 4 plus 4 plus 2 plus 1 plus	Bilirubin 1 4 mg %
M B	Chronic hepatitis	9/5	6 3+	7 3+	4 plus	Bilirubin 3 6 mg %
D K	Tuberculosis of bone, mild hepatitis, probably of serum type, (almost cured)	8/25	3	4 5	Neg	
E R	Tuberculosis of spine, hepatomegaly	8/25	6 5 3+	12 3+	4 plus	
T Z	Tuberculosis of lungs, homologous serum jaundice	8/25 9/2 9/11	7 4+ 7 3+ 6 3+	19 5 4+ 17 5 2+ 13 3+	4 plus 3 plus 1 plus	
H J	Infectious hepatitis (improved)	9/8	4 2+	14 3+	3 plus	
A C	Laennec's cirrhosis	5/2	1 5	3	Neg	T P, 61, A, 38, G 23, BSP, 40% left after 30 min, bilirubin, 0 3 mg % ind
B S	Cholangitic cirrhosis (Worse) (Very bad)	5/28 6/20 6/25 7/7 7/11 7/14	6 4 5 6 4 5 4 5 6	11 10 16 18 5 22 22	4 plus 4 plus 4 plus 4 plus 4 plus -	Bilirubin 1 1 mg % ind Bilirubin 7 5 mg % dir Bilirubin 4 3 mg % T P, 59, A, 31, G, 28, alkaline phosphatase, 58 U
		7/23 7/28 8/4	6 5 2+ 6 4 5	18 2+ 18 5 14	4 plus 4 plus 4 plus	1 P, 53, A, 24, G 29, bilirubin, 2 8 mg % dir Bilirubin 6 3 mg % dir
M M	Cirrhosis, diabetes, Kimmelstiel-Wilson syndrome	6/16	7	21	2 plus	T P, 58, A, 29, G, 29, BSP, 100% retention
F D	Osler's disease, cirrhosis	6/20 7/25	2 5 2	2 5 4	Neg 2 plus	BSP, 28% left after 45 min., T P, 54, A, 32, G, 22

dir Direct reacting and indirect reacting

T P Total protein A albumin G globulin

\*Hypertensive cardiovascular disease

†Arteriosclerotic heart disease

TABLE VII—CONT'D

PATIENT	DIAGNOSIS	DATE (1947)	DTT	TTT	CCF	OTHER FINDINGS
G D	Laennec's cirrhosis	7/16	75	95	2 plus	T 1 67 A 51 G, 18, bilirubin less than 0.1 mg %
		7/30	4	9	4 plus	
		9/20	6	11	3 plus	T P 66 A 23 G 43
			2+	2+		
		9/2	7	11	4 plus	
			7	3+		
I I	Cholangitis cirrhosis obstruction (adhesions)	7/28	1	10	4 plus	Bilirubin 2.2 mg % alkaline phosphatase 197 U
E C	Laennec's cirrhosis rheumatoid arthritis	7/10	1	10	4 plus	
I S	Laennec's cirrhosis congestive liver damage ASHD†	7/23	2	30	2 plus	
		8/1	30	80	4 plus	
			+	+		
		8/22	30	1	plus	
			+			
		9/12	2	55	Neg	
		9/22	30	6	Neg	
		9/26	5	55	1 plus	
W H	Laennec's cirrhosis carcinoma of biliary tract	8/1	20	40	4 plus	
M L	Laennec's cirrhosis biliary cirrhosis	9/8	90	205	4 plus	
			3+	4+		
		9/9	125	20	4 plus	T.P., 66 A 35 G 31
			4+	4+		
		10/15	12	210	4 plus	Cholesterol 198 esters, 136 mg %
			4+	4+		
N P	Cirrhosis carcinoma of liver	7/14	2	7	1 plus	
		9/9	2	6	2 plus	B51 70% left after 30 min T P 74 A 39 G 35 bilirubin 12 dir
M M	Biliary cirrhosis	10/20	3	7+	Neg	Cholesterol 198 esters, 135, bilirubin 37.5 mg % alkaline phosphatase 133 U
R B	Cirrhosis hepatitis	10/20	4	7	3 plus	
			2+	2+		
R K	Carcinoma of breast pm cirrhosis of liver and metastases		4	11	Neg	T P, 6 A, 34 G 26
			2+	2+		
I W	Carcinoma of head of pancreas complete obstruction with metastases		2	25	Neg	Bilirubin 14.2 mg %
C S	Obstructive jaundice malignant tumor	7/15	2	5	±	
E W	Obstructive jaundice carcinoma of head of pancreas	8/18	2	4	Neg	
		8/29	15	6	Neg	T 1 75 A, 31 G 42 bilirubin 45 mg % cholesterol 546 mg % esters 183 mg %

(Cont'd on next page)

TABLE VII—CONT'D

PA TIENT	DIAGNOSIS	DATE (1947)	D T T	T T T	C C F	OTHER FINDINGS
J L	Carcinoma of gall bladder, severe prolonged obstructive jaundice	8/29	3 5 4	10 5 9	Neg 1 plus	—
M F	Seminoma with metastases of liver, obstructive jaundice	9/5	1 5 3	8 6	Neg Neg	
M P	Lymphosarcoma, obstructive jaundice (10 days)	10/15	2	2 5	Neg	
E S	Chronic cholecystitis	9/22	4 +	5 +	Neg	
R D	Cholecystitis	8/11 8/18	3 3	8 5 9	Neg Neg	
C R	Pancreatitis, obstructive jaundice	6/18 6/25	2 2	1 5 2 5	Neg Neg	Alkaline phosphatase 29.4 U
	Day of operation	7/10	2 5	4	Neg	
W R	Jaundice after gastric operation, obstructive?	6/20 6/20 6/27 7/9	3 3 3 3	2 5 3 3 5 5 0	Neg Neg ± Neg	Bilirubin 17.5 mg % indir
A H	Diabetes, hepatomegaly, Kimmelstiel-Wilson syndrome	6/20 7/11	3 2	4 5 5	2 plus 1 plus	T P, 7.5, A, 5.1, G, 2.4

Table VII, which summarizes the cases of liver and biliary tract disease, shows the rather close parallelism of the tests with a few notable exceptions. Patient W R, following gastroenterostomy and vagotomy for duodenal ulcer and obstruction, presented a late postoperative picture of questionable obstructive jaundice possibly combined with mild parenchymal damage. Since no clear-cut diagnostic conclusion could be reached, the evaluation of the slight discrepancy in the tests is obviously impossible. Patients N P, C S, M F, and F W, patients with neoplastic disease, showed discrepancies between the dilution turbidity and the thymol turbidity tests; patient N P showed cirrhosis and carcinoma of the liver on surgical exploration.

It may be stated here that most of the discrepancies between dilution turbidity and thymol turbidity eventually fitted into three groups: neoplastic diseases, congestive heart failure, and, to a smaller extent, diabetes. In diabetes the thymol turbidity test is occasionally positive.

In Table VIII the cases of neoplastic disease are reviewed. Those belonging in this group but already reported in Table VII are not repeated but are included in the statistics. In eleven specimens out of sixty-five (excluding the rather uniform group of multiple myeloma) a discrepancy between dilution turbidity and thymol turbidity was noted, and in five, a discrepancy between the thymol turbidity test and the cephalin-cholesterol flocculation test.

TABLE VIII NEOPLASTIC DISEASE

PATIENT	DIAGNOSIS	DATE (1947)	D T T	T T T	C C F	OTHER FINDINGS
S D	Chronic lymphatic leucemia	7/7	3	10.5	4 plus	T P 59 A 4 G, 19
		7/28	2.5	9	4 plus	
		8/11	4	11	4 plus	
			3+	3+		
K B	Chronic lymphatic leucemia		2	2	Neg	
L J	Chronic lymphatic leucemia	7/16	0.5	1.5	-	
		9/12	1	1.5	Neg	
M G	Chronic lymphatic leucemia	7/30	1	7.5	4 plus	1 P 498 A 46 G 252
		8/4	0.5	7	plus	
				2+		
		8/22	0.5	7	3 plus	
				4+		
B G	Chronic myelogenous leucemia	8/18	1	2	Neg	1 P 58 A 38 G 2
		9/3	1	1.5	3 plus	
F C	Hodgkin's disease	6/30	2	4	Neg	
		8/8	2	4	Neg	
K W	Hodgkin's disease	7/9	1	2	0	
S	Hodgkin's disease	8/20	9	23	4 plus	T P 61, A 22 G 39
			3+	4+		
		9/10	8	24	2 plus	BSP 75% left after 30 min
			4+	4+		
		9/22	8.5	20	2 plus	
			4+	4+		
F S	Hodgkin's disease	9/26	4.5	11	1 plus	
			3+	2+		
S D	Carcinoma of colon with liver metastases	7/9	2	6.5	1 plus	
I R	Abdominal carcinoma with liver metastases	7/10	6	4	plus	
S G	Carcinoma of sigmoid with liver metastases	8/11	6	10	3 plus	
			2+	2+		
			5.5	8	2 plus	
			3+	2+		
G M	Carcinoma of colon liver enlargement (metastases?)	8/11	3	8	plus	
D R	Carcinoma of rectum	8/11	1	2.5	Neg	
P B	Carcinoma of colon	8/11	1.5	3	1 plus	
S C	Carcinoma of sigmoid	8/11	0.5	-	Neg	

(Contd on next page)

TABLE VIII—CONT'D

PA TIENT	DIAGNOSIS	DATE (1947)	D T T	T T T	C C F	OTHER FINDINGS
M R	Carcinoma of stomach	8/11	1	2	Neg	
T McQ	Carcinoma of rectum (after incompatible transfusion)	8/11	3 5 +	11 +	Neg	
S S	Carcinoma of stomach	8/11	1	4	Neg	
Z S	Carcinoma of esophagus	8/13	4	7	1 plus	
M K	Carcinoma of sigmoid	8/18	2 5	5 5	Neg	T P, 55, A, 28, G, 24, bil rubin, 0.1 mg %, alkaline phosphatase, 5 U
A W	Retroperitoneal tumor (cachexia)	7/25 8/1	2 1 5	3 3 5	2 plus 4 plus	T P, 69, A, 24, G, 45
O B	Hypernephroma	8/13	1 5	2	±	
L K <sup>e</sup>	Carcinoma of cervix	8/13	1 5	2 5	Neg	
S C	Carcinoma of prostate	9/17	1 5	1 5	Neg	Acid phosphatase 42 U
B B	Carcinoma of thyroid	6/18 6/25	1 2	2 2 5	Neg Neg	
N L	Bronchogenic carcinoma	5/23	4 5	12	2 plus	
L J	Carcinoma of lung	8/4 8/18	2 2	7 12 5 2+	3 plus 3 plus	T P, 498, A, 246, G, 252
G L	Bronchogenic carcinoma	8/13	2 5	2	2 plus	
I P	Bronchogenic carcinoma	8/13	1	2 5	Neg	
M S	Bronchogenic carcinoma	8/13	2	2 5	±	
A P	Carcinoma of breast	8/6	1 5	4	Neg	
R G	Carcinoma of breast	8/6	2	4	2 plus	
H H	Carcinoma of breast	8/6	1	3 5	Neg	
C B	Carcinoma of breast	8/6	0 5	1	Neg	
Y H	Carcinoma of breast	8/6	1 5	4	Neg	
C H	Carcinoma of breast	8/6	1	2 5	Neg	



TABLE VIII—CONT'D

PA TIENT	DIAGNOSIS	DATE (1947)	DTT	TTT	CCF	OTHER FINDINGS
R. W.	Carcinoma of breast	8/13 8/22	0.5 1.5	1 0.5	± Neg	
R. B.	Carcinoma of breast with bone metastasis	8/6	1.5	4.5	Neg	
D. C.	Multiple myeloma, p m widespread osseous involve ment, liver neg ative	5/28 7/23	2 2.5	1 0.5	Neg Neg	TP 106 A 24, G 82* TP 93 A 22 G 71
M. K.	Multiple myeloma	6/4 10/20	0.5 1	2 1	Neg Neg	TP 13 A 53 G 40*
M. W.	Multiple myeloma	6/4 7/28	0.5 0.5	1 1	Neg Neg	TP 70 A 50 G 20† TP 56 A 39 G 17
E. E.	Multiple myeloma	6/20	1.5	1	Neg	TP 538 A 397 G 441*

None of the e presented a Bence Jones proteinuria  
†Bence Jones proteinuria present

The small group of four cases of multiple myeloma presents an interesting feature insofar as, with one exception—a normal value—all the dilution turbidity and thymol turbidity values were low and all the cephalin cholesterol flocculations were negative. This is so, irrespective of the total protein and especially globulin levels which in three cases were high and in one low.

Table IX summarizes only those cases of congestive heart failure in which one or more of the tests were positive. In ten specimens out of fifty-seven taken from patients with congestive failure dilution turbidity and thymol turbidity did not conform; eight of them presenting an increased thymol turbidity and normal dilution turbidity and in fifteen there was an incongruity between the thymol turbidity test and the cephalin cholesterol flocculation.

Eleven specimens taken from nine patients with active tuberculosis, mostly advanced pulmonary tuberculosis (three of them were included in Table VII which reviews liver disease) showed ten positive dilution turbidity tests and ten positive thymol turbidity tests, all in the same specimens, and six positive cephalin cholesterol flocculation tests.

Some cases of special interest are presented in Table X. In accordance with the findings of Wertheimer and Stein<sup>14</sup> two cases of subacute endocarditis showed increased dilution turbidity and also conformed with their findings: a cold fraction was present in these sera. Carter and MacLagan<sup>1</sup> also have reported on the positivity of the thymol turbidity test in this disease. Liver damage though is hardly to be ruled out in subacute bacterial endocarditis. Positive liver tests have been reported in infectious mononucleosis by several authors.<sup>2-23</sup> It seems worth while emphasizing the increased values in the three patients with atypical pneumonia examined, a finding which we have not encountered in the few patients with other types of pneumonia examined. We intend to follow up this finding in an adequate series of atypical pneumonias.

TABLE IX CONGESTIVE HEART FAILURE

PATIENT	DIAGNOSIS	DATE 1947	D T T	T T T	C C F	OTHER FINDINGS
C R	RHD*	5/26 7/7	2 1	5 3	Neg Neg	Bilirubin 14 mg %
C S	RHD, hyper tension	6/23 7/25	2 3	3 4.5	1 plus 3 plus	T P, 76, A, 28, G, 48 BSP 49% left after 40 min
A J	RHD, chronic nephritis	8/20 9/8	0.5 1.5 1.5	1.5 2 1.5	± 2 plus Neg	
C W	RHD, p m nutmeg liver	7/23 8/1 8/18 8/25 9/3 9/12 9/26	1.5 2 1 0.5 2 1 1.5	2.5 3.5 4 4.5 3.5 3.5 3	Neg 3 plus Neg — 1 plus Neg 1 plus	
E MeC	RHD, p m chronic passive congestion, duo denal ulcer	8/13 9/3	2 2	5 3.5	4 plus 3 plus	T P, 69, A, 48, G, 21
H D	RHD	8/25 10/15	3 5	5.5 7 4+	Neg Neg	
J S	RHD	8/29	2	2	2 plus	
B S	RHD, pulmonary infarction	8/29 9/17	1.5 3 4	6 4.5 7	1 plus Neg 2 plus	Bilirubin 10 mg %
G S	RHD	9/29	7 4+	8	2 plus	
R B	ASHD,† RHD	6/2	2	5.5	2 plus	
M T	HCVD,‡ diabetes	7/11 8/18	0.5 2.5	5 4	Neg Neg	
H R	ASHD	8/25 9/8	1.5 1	1.5 2	Neg 1 plus	Bilirubin 33 mg %
D G	AHCVD§	9/5	2	6	Neg	
M B	ASHD	9/8	2.5	4.5	2 plus	
E S	AHCVD	9/9	3	3.5	Neg	
R T	AHCVD, diabetes	10/1	3.5	4	2 plus	
J G	AHCVD, diabetes	10/15	5 4+	8 3+	4 plus	
A M	Congenital heart disease	6/16	2.5	5	Neg	

\*Rheumatic heart disease

†Arteriosclerotic heart disease

‡Hypertensive cardiovascular disease

§Arteriosclerotic hypertensive cardiovascular disease

A group of eleven cases of rheumatoid arthritis presented a high proportion of positive dilution turbidity and thymol turbidity tests. From this point of view the disease is the subject of a study at the present time.

TABLE X MISCELLANEOUS DISEASES

PA TIENT	DIAGNOSIS	DATE (1947)	D T T	T T T	C C F	OTHER FINDINGS
L B	Subacute bacterial endocarditis	5/26	8	20	3 plus	2 plus cold fraction
R F	Subacute bacterial endocarditis	6/4 6/18 7/7	3 3 5 2	8 5 5 5	Neg 1 plus Neg	1 plus cold fraction Slight cold fraction No cold fraction
E E.	Subacute bacterial endocarditis ?	6/16	3	4	Neg	No cold fraction
D F	Infectious mono nucleosis	9/11 9/17	3 4 +	5 6 +	- - plus ±	
B B	Atypical pneu monia RHD	10/15 10/31	7 8 3+	9 11 2+	3 plus 3 plus	Cold agglutination 1 8+ Cold agglutination 1 250+
F H	Atypical pneu monia	7/14	4	8 5	2 plus	
S L	Atypical pneu monia	7/9 7/16	11 + 10	11 + 14	4 plus 4 plus	
M. C	Boeck s sarcoid liver enlarge ment	7/9	8 2+	17 2+	4 plus	
F B	Diffuse vascular disease	7/25 9/8	5 + 3 2+	9 + 4.5 2+	2 plus 4 plus	1 P 59 A 46 G 13 bil rubin 02 mg %

\*Rheumatic heart disease.

Positive flocculation reactions (twenty four hour reading) are marked plus to 4 plus according to their degree where observed but in the first few weeks of this work insufficient attention was paid to this phenomenon. No statistical conclusions, therefore, are drawn from them. We could not in contrast to Neeffe<sup>24</sup> observe an advantage of the thymol flocculation test over the thymol turbidity test as a more sensitive indicator for liver damage we found it positive only whenever the thymol turbidity was over 4. We made the same observation for the dilution flocculation and looked at the flocculation reactions only for their confirmatory value.

## DISCUSSION

The behavior of the dilution turbidity toward NaCl and distilled water and toward lipid extraction and addition respectively and its negative result in tests performed on rabbits' blood and on immune globulin demonstrate its close relationship to the thymol turbidity test. This relationship furthermore is borne out by the clinical data. We even venture to suggest that a considerable part of the precipitation in the thymol turbidity test is due to the diluting effect of the distilled water. Certain differences however (as both the rapid deterioration of the former fraction and a sizable number of discrepancies in the clinical material indicate) make it appear improbable that the respective fractions responsible for the two tests are identical they may overlap though and be very close to each other.

In accordance with Recant, Charzaff and Hanger,<sup>7</sup> Watson and Rappaport,<sup>25</sup> Neeffe<sup>24</sup> Mateer and co workers,<sup>26, 2</sup> Havens and Marck<sup>28</sup> and Cohen

and Thompson,<sup>6</sup> we conclude from our experience that the cephalin cholesterol flocculation indicates a different serum pattern from the one responsible for the thymol turbidity test and, for that matter, for the dilution turbidity test.

Reviewing the clinical results, a few findings require further comment. Among the nine cases in which the dilution turbidity alone was positive, there were three in which the thymol turbidity value was 4, a value considered positive by Mateer and co-workers,<sup>26</sup> one was a case of congestive heart failure, and one a probable intra-abdominal malignant tumor. Among the twenty-one cases in which the thymol turbidity test alone was positive, four represented malignant diseases, five congestive failure, and five diabetes. Of six specimens in which the dilution turbidity test and the cephalin-cholesterol flocculation test were positive together, four had a thymol turbidity of 4 and one of 3.5, and one was from a patient with a malignant disease. The group where the thymol turbidity test and the cephalin-cholesterol flocculation test were both positive together (four specimens) is made up of cases of congestive failure and neoplastic disease with the single exception of one case of diabetes.

Irrespective of these considerations, when summarizing our figures as given, it becomes evident that the association of findings of the dilution turbidity and thymol turbidity tests is not merely a matter of chance, these two tests are positive together in 124 specimens, the dilution turbidity test and cephalin cholesterol flocculation together in 96. Since the standard error of difference is 4.95, the difference between both series of findings exceeds by five and one half fold the standard error of difference.

The majority of the findings belongs to the group in which all the tests were positive. Most diseases, especially those of the liver, which cause profound protein disturbances apparently are able to produce positive tests in several groups. In a severe parenchymatous disease of the liver such as infectious hepatitis, the results of most of the valuable liver tests may well be expected to be positive, together at the same time or at least following each other in different phases of the disease, whether the tests belong to the hepatocellular or the cholangiolar type (Watson and Hoffbauer<sup>29</sup>).

As Cantarow<sup>30</sup> and others have shown, other diseases of the liver are less likely to produce a large number of different positive tests simultaneously. Hanger has pointed out repeatedly that some cases of malignant diseases of the liver, primary or metastatic, usually fail to produce a positive cephalin cholesterol flocculation. Watson and Rappaport<sup>25</sup> mention in their table of disagreements between cephalin-cholesterol flocculation and the thymol turbidity test four cases where, on the contrary, the cephalin-cholesterol flocculation was 1 plus to 2 plus and the thymol turbidity test was negative.

Congestive failure of the heart may attack the liver in various ways and so produce varying functional effects expressing themselves in incongruous results of tests.<sup>21-31</sup> It is conceivable that in both groups of cases, neoplastic diseases on the one hand and the congestive damage of the liver on the other, different cell units may be affected in different degrees and ways, and, as for the first category, the damage might not necessarily be in the liver.

The determination of the dilution turbidity and flocculation may prove itself useful for several reasons. It supplies, in our experience, most of the information provided by the thymol turbidity and flocculation test. Its departure from the thymol test may, in addition, turn the attention to one of the disease groups mentioned in which this occurs, and it may be helpful in the classification of a case of striking hyperglobulinemia. The dilution turbidity test is easily carried out and, after some experience in its use has been acquired, may even be employed as a bedside procedure.

#### SUMMARY

The procedure of diluting serum with distilled water was carried out and the resulting turbidity was read photometrically. This dilution turbidity test was compared with Mucлагan's thymol turbidity test and Hanger's cephalin cholesterol flocculation test.

It was noted that at a dilution of 1:15 the dilution turbidity closely parallels the thymol turbidity.

Experimental evidence confirms this observation of a close relation between these two tests, the technique of the procedure, the behavior of the dilution turbidity toward water and sodium chloride, its values before and after lipid extraction of the sera, its behavior with rabbits' blood and human immune serum globulin, and the fact that it flocculates within twenty-four hours when present in increased amounts, all put the dilution turbidity test beside the thymol turbidity test rather than the cephalin cholesterol flocculation test.

Conforming with evidence obtained by others, a certain difference in mechanism between the thymol turbidity test and the cephalin cholesterol flocculation test also is confirmed by our experiments and clinical experience.

There is, however, an obvious difference in the results of the thymol and dilution tests in a small number of cases as well as in the experimental results. The discrepancies between the two tests as observed in clinical cases usually fall into the categories of neoplastic disease, congestive heart failure, and diabetes. The serum components responsible for the two respective tests therefore cannot be identical but may overlap.

The dilution turbidity test may serve as a simple test apparently giving most of the information supplied by the thymol turbidity test. It may further more confirm and occasionally modify the result obtained in a thymol test.

I wish to thank Dr. E. Wertheimer for arousing my interest in this field, Mr. G. Ross for his kind help and interest, Dr. L. Leiter, Dr. F. M. Hanger, and Dr. L. Recant for valuable suggestions, and the house and laboratory staffs of Montefiore Hospital for their fine cooperation.

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# EXPERIMENTAL STUDIES ON THE INTRAVENOUS INJECTION OF A FAT EMULSION INTO DOGS

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IT IS important to be able to provide complete parenteral nutrition for human beings. To accomplish this it seems necessary to have a stable, nontoxic fat emulsion suitable for administration by vein. The literature contains numerous reports<sup>1-7</sup> recording the efforts of other investigators in this direction. From studying these reports it is apparent that none of the emulsions thus far prepared is entirely free from toxic effects.<sup>5-7</sup> Emboli, anemia, or liver damage appear to be the most common complications arising from the injection of intravenous fat.

The following is an account of our most successful experiences in attempting to prepare a stable, nontoxic fat emulsion suitable for intravenous use.

## EXPERIMENTAL PROCEDURE AND RESULTS

The problem may be divided into three parts: (1) preparation of a uniform, stable, finely divided fat emulsion, (2) a study of the physiologic effects of the emulsifying agents and of the complete fat emulsion, and (3) evidence concerning the utilization of fat contained in the emulsion.

### *Preparation of a Uniform Stable Fat Emulsion.—*

The emulsifying agents studied included Tweens, Spans,<sup>†</sup> soya bean phosphatides,<sup>‡</sup> mono- and diesters of glycerol phosphate esters of long chained hydrocarbon alcohols, gums, plasma, and bile salts.

Many substances were tried alone or in combination with one another. The oils used were coconut, corn, or butter oil. The fat soluble agents were dissolved in the fat and the water soluble substances in water prior to mixing the water and oil together. Dextrose, Karo syrup, or dextrans were also dissolved in the aqueous phase in some instances. Preliminary studies on emulsification usually were made with a hand homogenizer. If these experiments appeared promising as judged by the particle size and ease of emulsification a larger batch of material was emulsified by means of a Cherry Burrell Viscolizer.<sup>§</sup>

The following procedure and substances were finally adopted as yielding a satisfactory emulsion containing a minimum of emulsifying agents. Fresh sweet butter was melted and the oil separated by centrifugation, after which it was washed three times using four volumes of hot distilled water for each washing. Five grams of Span 20 and 4 Gm of pure soya phosphatides (Asolectin) were added to every 100 Gm of butter oil.

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<sup>†</sup>Tweens and Spans are surface active agents produced by the Atlas Powder Company, Wilmington, Del.

<sup>‡</sup>Purified Soya Phosphatides, Asolectin, a product of Associated Concentrates, Inc., Atlanta, Ga.

<sup>§</sup>The authors wish to acknowledge their indebtedness to the Cutter Laboratories, Berkeley, Calif., for the funds used to purchase the Viscolizer.

The mixture was heated and mixed on a steam bath until dispersion was complete. One hundred grams of dextrose and 1 Gm of sodium cholate\* were dissolved in sufficient water to make 883 ml of solution. The butter oil and dextrose cholate solution were then stirred together and poured into the homogenizer. Usually 10 to 15 liters of emulsion were prepared at one time. This amount required four to five pounds of butter.

The mixture was passed through the Viscolizer five times at a pressure of 3,000 to 4,000 pounds per square inch †. At the end of this procedure the milky white emulsion was filtered through gauze into 500 ml Erlenmeyer flasks (400 ml to a 500 ml flask) and the flasks were stoppered with cotton. The flasks of emulsion were autoclaved twice within twenty-four hours at 10 pounds pressure (113 to 114° C) for ten minutes. Part of each batch was incubated for forty-eight hours at 37.5° C, and 1 ml was added to fluid thioglycollate broth and cooked meat broth media. The inoculated media were incubated for forty-eight hours, then streaked on blood agar plates and incubated aerobically and anaerobically. The bacteriologic tests were negative when the emulsion was sterilized as described ‡. The pH of the emulsion after autoclaving was 6.3 to 6.5. The flasks of emulsion were then stored at room temperature till used. The particle size of the emulsion, measured by means of an objective micrometer, ranged from 0.5 to 2  $\mu$  in diameter and was quite uniform. Emulsion allowed to stand for two months or more in the laboratory showed little change in gross or microscopic appearance. Freezing and thawing caused a large portion of the fat to separate from the emulsion. Some increase in particle size and a tendency to cream result from long standing.

TABLE I

CONTENTS	GM /KG BODY WT	CAL /KG BODY WT	% CALORIES
Sucrose	11.1	45.4	49.0
Casein	4.4	18.04	19.4
Yeast	0.9	-	-
Cellophane	0.9	-	-
Salt mixture*	0.4	-	-
Lard	3.2	29.3	31.6
Total	20.9	92.7	
Vitamin A	250 units		
Vitamin D	36 units		

\*Wesson's salt mixture<sup>9</sup> was used in all the synthetic diets.

The effects of the emulsifying agents were studied by preparing emulsions identical in every respect with those just described except that they contained no butter oil. The acute experiments were carried out on dogs under Nembutal anesthesia. The longer term feeding experiments were done on animals that had been dewormed and had undergone preliminary observations in the laboratory for several weeks prior to use. These animals were confined in cages which permitted a quantitative collection of urine. Fifty ml of 15 per cent (by volume) hydrochloric acid and 1 ml of 10 per cent thymol solution in 95 per cent alcohol were used as preservatives, and urine was collected for a three-day period weekly during experimentation.

The partly synthetic diet (Table I) supplying ninety-three calories per kilogram per day was fed to all animals on metabolic experiments during the control period. A low carbohydrate diet (Table II) was fed to the dogs injected with emulsifying agents (in 10 per cent dextrose), while the fat injected animals received a fat free low carbohydrate diet (Table III). However, the animals received isocaloric diets throughout the experimentation.

\*Prepared from cholic acid obtained from G. H. Breon & Co. Inc., Kansas City, Mo.

†During homogenization the temperature of the solution rises from 27° C to approximately 45 to 50° C.

‡The tests for sterility were performed by Dr. Evelyn P. Tilden of the Bacteriology Department of the Dental School, Northwestern University.



TABLE II LOW CARBOHYDRATE DIET

CONTENTS	GM /KG BODY WT	CAL /KG BODY WT	% CALORIES
Sucrose	7.9*	32.5	49.0
Casein	4.4	18.0	19.4
Yeast	0.9	-	-
Cellophane	0.90	-	-
Salt mixture	0.42	-	-
Lard	1.1	29.7	31.6
Total	16.6	79.8	
Vitamin A	250 units		
Vitamin D	36 units		

3.146 Gm per kilogram as dextrose were given by vein furnishing 17.9 calorie

In the longer term experiments the animals were injected while secured on their sides on a canvas frame. The emulsion or emulsifying agents were injected six days per week through a 21 gauge needle into the veins of alternate legs. A rate of approximately 15 ml per minute for a dog weighing 12 kilograms was used in all experiments. Sterile precautions were observed insofar as this was possible. Injection was by means of a plastic tubing secured in the external jugular vein in some instances.

TABLE III FAT FREE LOW CARBOHYDRATE DIET

CONTENTS	GM /KG BODY WT	CAL /KG BODY WT	% CALORIES
Sucrose	7.93*	32.5	49.0
Casein	4.4	18.0	19.4
Yeast	0.902	-	-
Cellophane	0.90	-	-
Salt mixture	0.42	-	-
Lard	—*	-	31.6
Total		50.5	
Vitamin A	250 units	-	-
Vitamin D	36 units		

3.146 Gm per kilogram body weight as dextrose and 3.146 Gm per kilogram body weight as fat were given by vein furnishing 42 calories

*Immediate Effects of Injection of the Emulsifying Agents or Fat Emulsion on Dogs*—The effects of infusing the emulsifying agents or fat emulsion on the arterial blood pressure and on the flow of thoracic duct lymph, hepatic bile, and urine were determined\*. After a one half to one hour control period infusion was begun and continued at the rate of 0.12 ml per kilogram per minute for the next four hours, after which observations were continued for another hour or longer. Table IV summarizes the results on dogs that received the emulsifying agents (three dogs) or the fat emulsion (five dogs). Blood pressures were not included for in no instance were they altered in either group of dogs. Bile flow

The procedure for these measurements was briefly as follows. An animal was anesthetized with Nembutal (1 grain for every 5 pounds) and secured on his back. A mercury manometer was connected with the left carotid artery by thick walled rubber tubing and a glass cannula filled with citrate solution. Through a right rectus incision the common bile duct was exposed and cannulated and the cystic duct ligated. A small bore rubber tube was connected to the cannula and brought to the outside by means of a stab wound in the right side. The thoracic duct was exposed in the left supraclavicular fossa and cannulated after which the lymph was collected in test tubes containing a small quantity of potassium oxalate. The ureters were cannulated at the brim of the pelvis and urine was collected from small bore rubber tubes attached to the glass cannulas and exteriorized through perineal stab wounds.

TABLE V EFFECT OF FAT EMULSION, EMULSIFYING AGENTS, AND DEXTROSE ON VOLUME OF THORACIC DUCT LYMPH

PERIOD OF COLLECTION		FAT EMULSION												EMULSIFYING AGENTS												DEXTROSE 10%		
RELATION TO INJECTION	DURATION OF COLLECTION (MIN.)	DOG 905			DOG A			DOG B			DOG C			AVERAGE			DOG 910			DOG 4			AVERAGE			DOG S		
		TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN	TOTAL C C	PER C C	MIN
Before	60	19.6	0.33	7.4	0.12	0.28	0.20	0.33	0.33	0.33	0.33	0.33	0.33	16.0	0.25	0.25	20.4	0.34	0.34	20.0	0.33	0.33	20.2	0.34	0.34	16.0	0.27	0.27
During	60	31.0	0.52	12.5	0.21	0.21	0.25	0.41	0.41	0.41	0.41	0.41	0.41	35.8	0.60	0.60	31.0	0.52	0.52	23.0	0.38	0.38	27.0	0.45	0.45	22.0	0.37	0.37
During	60	33.4	0.56	20.5	0.34	0.42	0.70	0.32	0.53	0.53	0.53	0.53	0.53	32.7	0.53	0.53	19.5	0.33	0.33	30.0	0.50	0.50	25.0	0.42	0.42	29.0	0.48	0.48
During	60	45.0	0.66	36.0	0.40	0.31	0.52	0.75	0.47	0.47	0.47	0.47	0.47	47.0	0.59	0.59	41.5	0.35	0.35	82.0	0.75	0.75	62.0	0.55	0.55	53.0	0.44	0.44
After	60	37.5	0.62	22.0	0.37	0.21	0.35	0.60	0.60	0.60	0.60	0.60	0.60	35.5	0.49	0.49	14.0	0.23	0.23	63.0	1.05	1.05	38.5	0.64	0.64	25.0	0.42	0.42
After	60	16.0	0.27	14.0	0.33	0.19	0.32	0.32	0.32	0.32	0.32	0.32	0.32	30.0	0.50	0.50	0.50	0.12	0.12	41.0	0.68	0.68	24.0	0.40	0.40	18.0	0.30	0.30
After	60	0	0	20.0	0.17	0.37	0.31	0.102	0.85	0.85	0.85	0.85	0.85	53.0	0.44	0.44	0.44	0.25	0.25	52.0	0.87	0.87	33.0	0.55	0.55	30.0	0.25	0.25

tion The nitrogen balance was positive throughout the injection period as it was during the period of control observations for both groups of dogs. These results are shown in Figs 1 and 2

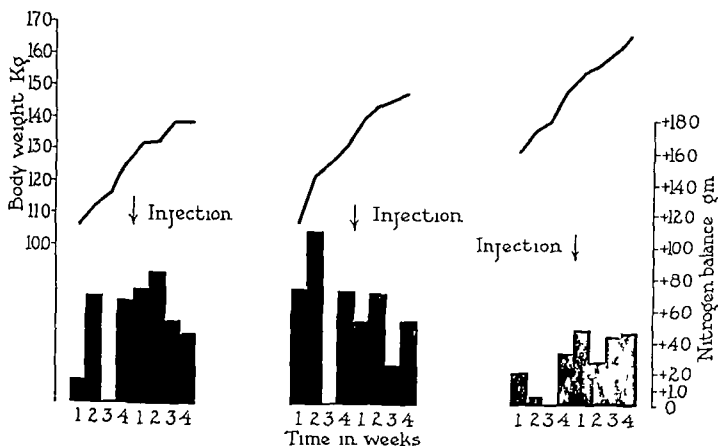


Fig 1—Showing a nitrogen retention (columns) and gain in body weight during daily injections of emulsifying agents similar to those during the control periods

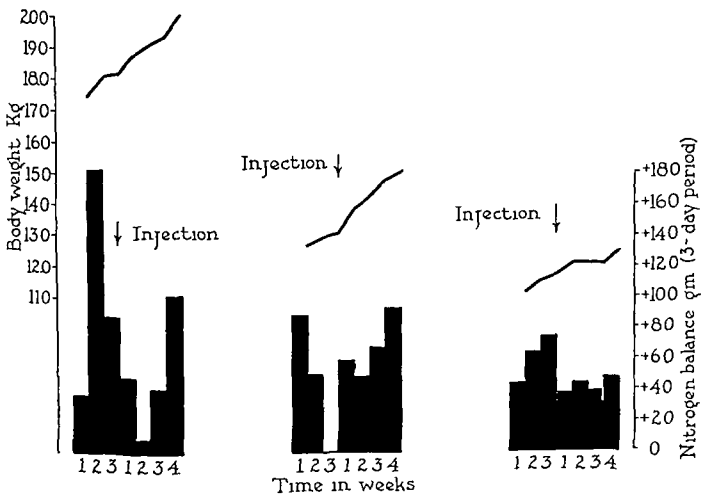


Fig 2—Showing a nitrogen retention (columns) and gain in body weight during periods of daily injections of the fat emulsion similar to those during the control periods

TABLE VI EFFECT OF FAT EMULSION AND OF EMULS

PERIOD OF COLLECTION		DOG A								DOG B			
RELATION TO INJECTION	DURA TION OF COLLEC TION (MIN)	TOTAL FATTY ACIDS		TOTAL CHOLES TEROL		PHOSPHO LIPIDS*		TOTAL FATTY ACIDS		TOTAL CHOLES TEROL		RELATION TO INJECTION	DURA TION OF COLLEC TION (MIN)
		CON (MG (%))	OUT PLT (MG)	CON (MG (%))	OUT PUT (MG)	CON (MG (%))	OUT PUT (MG)	CON (MG (%))	OUT PUT (MG)	CON (MG (%))	OUT PUT (MG)		
Before	60	576	43	105	7 8	150	11 1	427	85 4	143	10 5	Before	60
During	60	484	61	122	16 0	160	20 0	534	131	195	15 5	During	60
During	60	233	48	72	14 2	105	21 6	762	244	119	10 5	During	60
During	90	298	107	64	23 0	108	39 0	844	634	195	15 5	During	90
After	60	349	77	67	14 8	117	25 8	—	—	134	10 5	After	60
After	60	342	48	69	9 7	120	16 8	907	643	148	10 5	After	60
After	90	312	62	69	13 8	122	24 4	785	800	153	10 5	After	90

\*Phospholipids were obtained by multiplying phosphorus by 25

TABLE VII HEMATOLOGIC EFFECT OF INJECTING EMULSIFYING AGENTS ALONE

DOG	PROCEDURE	WEEK	DIET	AMOUNT GIVEN I V	BLOOD			HEMATO CRIT (%)
					HB (GM %)	R B C	W B C	
3	Control	1	Normal		16 0	7 30	9,450	51
		2	synthetic		15 5	6 48	10,300	41
		1	Low carbo	360 c c	15 0	6 68	14,450	51
		2	hydrate	0 5% in 10%	15 0	6 80	15,300	99
4A	Emulsi fying agents	3	diet	dextrose daily	15 0	6 12	16,400	49
		4			16 8	7 11	17,900	—
		1	Normal		14 0	6 10	13,300	40 1
		2	synthetic		14 5	6 35	11,200	40
4B	Emulsi fying agents	1	Low carbo	380 c c	15 0	6 38	14,500	41
		2	hydrate	1% in 10%	14 8	5 96	14,800	40
		3	diet	dextrose daily	12 0	4 62	18,150	35 3
		4			12 0	4 64	20,400	32
4C	Emulsi fying agents	1	Normal		15 8	6 63	16,000	41
		2	synthetic		16 2	6 90	13,000	43
		1	Low carbo	415 c c	16 5	6 68	13,800	44
		2	hydrate	1% in 10%	16 5	6 67	14,600	42 3
4D	Emulsi fying agents	3	diet	dextrose daily	14 5	5 76	19 400	40
		4			14 0	5 42	17,650	40
		1	Normal		16 5	6 56	16,800	43 3
		2	synthetic		16 5	6 40	15,000	43
4E	Emulsi fying agents	1	Low carbo	460 c c	17 0	6 76	18,800	44 3
		2	hydrate	1% in 10%	16 2	6 25	16,000	44 3
		3	diet	dextrose daily	16 5	6 26	19,000	44 3
		4			15 5	5 83	17 950	41 3

## STUDIES ON LIPID CONTENT OF THORACIC DUCT LYMPH

EMULSION										EMULSIFYING AGENTS					
DOG C										DOG Z					
PHOSPHO LIPIDS		TOTAL FATTY ACIDS		TOTAL CHOLESTEROL		PHOSPHO LIPIDS		TOTAL FATTY ACIDS		TOTAL CHOLESTEROL		PHOSPHO LIPIDS		TOTAL FATTY ACIDS	
CON	OUT	CON	OUT	CON	OUT	CON	OUT	CON	OUT	CON	OUT	CON	OUT	CON	OUT
(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)	(MG)
8	45.6	312	53	75	12.7	85	14.8	1,730	344	100	10	98	19.6		
10	50.3	360	274	86	64.5	110	82.0	1,216	280	90	18.4	141	32.4		
17	63.1	437	184	83	34.8	104	43.6	824	247	78	23.4	111	33.2		
8	161	444	198	85	26.4	121	37.6	440	360	63	51.6	98	72.1		
18	146	608	136	94	19.7	131	27.5	328	206	54	14.0	103	68.0		
16	183	680	130	97	18.9	180	34.5	258	106	57	23.4	117	48.0		
10	250	800	296	90	33.2	195	72.1	264	139	55	28.6	93	48.4		

TABLE VIII. HEMATOLOGIC EFFECT OF FAT EMULSION INFUSION

DOG	PROCEDURE	WEEK	DIET	AMOUNT GIVEN LV	BLOOD			
					HG (GM %)	RBC	WBC	HEMATO CRIT (%)
	Control	1	Normal synthetic diet		15.5	5.33	9,450	53
		2			16.0	6.90	13,150	53
	Fat in jection	1	Fat free low CHO diet	331 cc 10% fat in 10% dextrose daily	16.0	6.37	17,650	52
		2			16.0	6.79	15,650	53
		3			16.0	6.40	19,250	50
		4			16.2	6.78	18,550	-
	Control	1	Normal synthetic diet		14.5	6.18	8,900	49
		2			15.0	6.40	13,750	50
	Fat in jection	1	Fat free low CHO diet	328 cc 10% fat in 10% dextrose daily	15.0	6.41	14,500	50
		2			15.0	6.54	13,500	51
		3			15.5	6.73	13,000	49
		4			16.4	7.01	12,200	-
	Control	1	Normal synthetic diet		16.0	6.52	18,000	39
		2			15.8	6.53	17,000	40
	Fat in jection	1	Fat free low CHO diet	570 cc 10% fat in 10% dextrose daily	16.0	6.63	20,000	40
		2			15.5	6.02	11,750	40
		3			16.0	6.32	18,000	43
		4			15.2	6.25	10,600	41
	Control	1	Normal synthetic diet		16.2	6.79	16,000	44
		2			17.0	7.76	12,700	48
	Fat in jection	1	Fat free low CHO diet	408 cc 10% fat in 10% dextrose daily	17.0	7.24	9,300	45
		2			16.0	6.56	7,500	45
		3			16.0	6.11	13,350	45
		4			15.5	6.37	11,500	43
	Control	1	Normal synthetic diet		16.9	7.29	25,000	45
		2			17.2	7.58	16,450	
	Fat in jection	1	Fat free low CHO diet	363 cc 10% fat in 10% dextrose daily	17.2	7.21	16,300	46
		2			17.0	7.02	16,250	45
		3			16.5	6.50	15,000	42
		4			14.0	6.09	19,750	41.5

The total fat injection for each dog was as follows: Dog 1 714 Gm, Dog 7 1,398 Gm, Dog 8 979 Gm, Dog 9 871 Gm, Dog 10 714 Gm, Dog 18 78 Gm.

*Blood chemistry and liver function* There was no change in total plasma protein or in plasma nonprotein nitrogen in animals infused either with emulsifying agents or fat emulsion (Tables IX and X). There was no evidence of liver damage as judged by the rose bengal clearance test and by serum phosphatase determination in either group of dogs (Tables IX and X). One dog (Dog 9) showed a slight elevation of serum phosphatase. The urinary albumin and sugar tests were all negative throughout.

TABLE IX EFFECT OF INJECTION OF EMULSIFYING AGENTS ON LIVER FUNCTION, PLASMA PROTEINS, AND NONPROTEIN NITROGEN

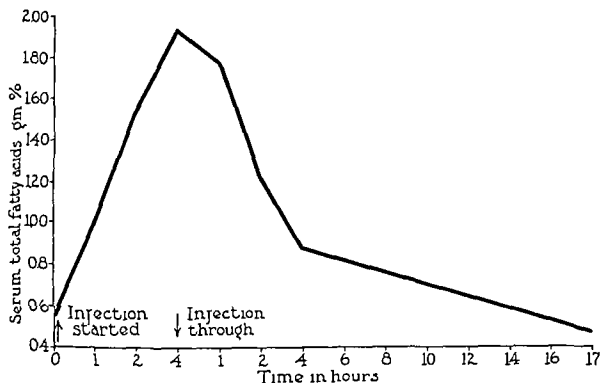
DOG	PRO CEDURE	WEEK	DIET	AMOUNT GIVEN I V	TOTAL PLASMA PRO TEINS (GM %)	PLASMA N P N (MG %)	ROSE BENGAL CLEAR ANCE	SERUM PHOS PHATASE
9A	Control	1	Normal					
		2	synthetic		5.51	27.7	100	2.50
		3	diet		5.80	27.9	107	2.71
	Emulsifying agents	4			5.78	29.0	100	2.40
		2	Low CHO	380 c c	5.80	28.2	109	2.90
		4	diet	1% in 10% dextrose daily	6.16	35.2	112	2.50
9B	Control	1	Normal		7.30	30.0	119	2.10
		2	synthetic		7.27	30.3	109	2.30
		3	diet		7.39	32.4	109	1.71
	Emulsifying agents	4			7.23	36.0	106	1.85
		2	Low CHO	415 c c	6.97	27.0	119	1.50
		4	diet	1% in 10% dextrose daily	7.09	37.1	126	1.40
9C	Control	1	Normal		6.78	27.1	113	2.00
		2	synthetic		6.90	32.1	100	2.64
		3	diet		6.65	28.8	106	2.69
	Emulsifying agents	4			7.34	35.0	109	2.53
		2	Low CHO	460 c c	7.05	25.2	115	1.3
		4	diet	1% in 10% dextrose daily	7.31	30.0	118	1.66

Post-mortem examination at the end of the four-week injection period for dogs infused both with emulsifying agents and fat emulsion demonstrated no gross abnormalities. Microscopically, Dogs 9B and 9C showed normal structure of liver and kidney, and no increase in fat deposition in these organs was demonstrated. In the fat injected animals, both Dogs 7 and 8 showed normal lungs, and no abnormalities were observed in the liver and kidney sections of Dog 8. However, in Dog 7 stainable fat granules were present in the liver and Kupffer cells, especially in the periportal areas. In the kidney section of this dog there was a diffuse, faintly positive fat stain in the cytoplasm of the distal convoluted tubules. No globules of fat were demonstrated in the glomeruli. The hematoxylin and eosin stained sections of this kidney revealed an occasional mass of pink material compressing the glomeruli and occupying a portion of the space within Bowman's capsule. No similar material was found elsewhere in the kidney.

TABLE X EFFECT OF INJECTION OF FAT EMULSION ON LIVER FUNCTION PLASMA PROTEIN AND NONPROTEIN NITROGEN

DOG	PROCEDURE	WEEK	DIET	AMOUNT GIVEN IV	TOTAL PLASMA PROTEINS (GM %)	PLASMA N P N (MG %)	ROSE BENGAL CLEAR ANCE	SERUM PHOS- PHATASE
7	Control	1	Normal		5.88	32.9	114	1.70
		2	synthetic		—	—	—	—
		3	diet		5.64	32.4	107	1.70
	Fat in	2	Fat free	570 cc	5.64	34.8	103	—
	jection	4	low CHO diet	10% fat in 10% dextrose daily	5.96	26.2	117	2.94
8	Control	1	Normal		5.9	33.4	107	0.83
		2	synthetic		—	—	—	—
		3	diet		6.24	30.1	102	1.24
	Fat in	2	Fat free	408 cc	6.17	31.8	106	—
	jection	4	low CHO diet	10% fat in 10% dextrose daily	6.16	29.2	105	2.29
9	Control	1	Normal		6.12	25.7	115	2.62
		2	synthetic		—	—	—	—
		3	diet		5.78	26.0	120	2.14
	Fat in	2	Fat free	363 cc	5.82	30.7	126	—
	jection	4	low CHO diet	10% fat in 10% dextrose daily	5.90	25.7	113	4.16

— Not determined

Fig. 3—Showing the average increase and rate of removal of fatty acids from the serum of two dogs (Dogs M and M<sub>2</sub>) injected repeatedly with a 10 per cent fat emulsion (average of all the fatty acid data in Table XI)

The rate of disappearance of intravenously injected fat The fasting serum lipids and the rate of fat disappearance following single and repeated injections were also determined. Total serum fatty acids, cholesterol, and phospholipids were estimated using the methods described for the lymph lipids.

Two dogs (Dogs M1 and M2) were given daily intravenous injections of fat emulsion for two weeks. The amount of fat emulsion given was based on body weight, as was that for the dogs under nitrogen balance study, and the general stock diet was fed ad libitum. Serum lipids were determined during the first day and at the end of the first and second weeks of injections. The total fatty acids concentration of the serum was markedly increased during fat injection. The increase declined as soon as the injection was discontinued or shortly thereafter and returned to normal within seventeen hours. The elevation

TABLE XI. EFFECT OF REPEATED INJECTIONS OF FAT EMULSION ON SERUM LIPIDS

DOG	TIME OF BLOOD SAMPLING	SERUM LIPIDS (MG %)							
		TOTAL FATTY ACIDS			TOTAL CHOLESTEROL			PHOSPHOLIPIDS	
		FIRST INJECTION	END OF 1ST WK. OF INJECTION	END OF 3RD WK. OF INJECTION	FIRST INJECTION	END OF 1ST WK. OF INJECTION	END OF 3RD WK. OF INJECTION	FIRST INJECTION	END OF 1ST WK. OF INJECTION
M1	Before injection	654	467	444	247.5	208.0	165.6	390	240
	First hour during injection	1212	875	960	227.5	196.0	155.2	376	280
	Second hour during injection	2192	1260	1432	230.0	200.0	160.0	415	276
	End of injection*	2411	1686	2006	241.0	227.5	165.5	438	312
	First hour after injection	1831	—	1625	247.0	225.0	175.2	445	298
	Second hour after injection	1122	806	1323	228.0	214.0	173.2	388	307
	Fourth hour after injection	765	519	857	200.8	210.0	165.6	329	307
	Next morning	618	366	528	201.8	195.0	156.0	329	260
M2	Before injection	664	507	502	218.8	250.0	215.2	336	350
	First hour during injection	1192	880	828	192.8	236.0	206.4	298	320
	Second hour during injection	1920	1121	1332	212.0	241.5	212.0	346	325
	End of injection*	2527	1376	1502	228.0	260.5	222.0	387	370
	First hour after injection	1791	824	1409	226.0	241.5	228.0	357	362
	Second hour after injection	1577	635	1323	220.0	234.0	224.0	340	362
	Fourth hour after injection	1241	524	1372	206.8	232.0	212.0	350	325
	Next morning†	568	394	521	206.8	227.5	199.2	357	319

\*Three to four hours

†About seventeen hours after previous injection was discontinued

‡Phospholipids were obtained by multiplying phosphorus by 25

of serum phospholipids which occurred toward the end of injection was slight but consistent. The serum total cholesterol demonstrated an initial fall at the end of the first hour during injection, after which it was increased toward the normal level or slightly above. The second fall occurred one hour or so after injection was discontinued. The results are shown in Table XI and Fig. 3.

Another two animals (Dogs R and H) were given a single intravenous injection of fat emulsion. The serum lipid changes were essentially the same as those of Dogs M1 and M2. When the same animals (Dogs R and H) were given a single injection of emulsifying agents alone (no fat) only a slight change in serum lipids occurred (Table XII).



TABLE XII. COMPARISON OF THE EFFECT OF FAT EMULSION AND OF EMULSIFYING AGENTS ON SERUM LIPIDS

DOG	TIME OF BLOOD SAMPLING	FAT EMULSION			EMULSIFYING AGENTS		
		TOTAL FATTY ACIDS (MG %)	TOTAL CHOLESTEROL (MG %)	PHOSPHOLIPIDS† (MG %)	TOTAL FATTY ACIDS (MG %)	TOTAL CHOLESTEROL (MG %)	PHOSPHOLIPIDS (MG %)
I	Before injection	480	184.0	214	372	158	230
	First hour during injection	1421	214.0	262	419	1.8	250
	Second hour during injection	2228	138.2	303	449	154	250
	End of injection*	3071	210.0	435	496	145	14
	First hour after injection	3298	236.0	320	334	154	254
	Second hour after injection	2420	235.8	332	528	145	288
	Fourth hour after injection	1073	222.5	308	561	142	280
	Next morning†	477	188.8	298	372	158	241
II	Before injection	762	261.5	485	533	223.5	328
	First hour during injection	1481	240.0	467	533	223.5	318
	Second hour during injection	1738	216.8	385	53	219.0	330
	End of injection*	3750	275.5	507	592	204.0	365
	First hour after injection	3544	314.0	575	579	220.5	334
	Second hour after injection	2509	306.5	562	588	202.0	334
	Fourth hour after injection	1917	298.0	475	570	191.5	329
	Next morning†	688	260.0	435	542	223.5	345

Three to four hours

† About twenty one hours after injection was discontinued. Accidental feeding.

‡ Phospholipids were obtained by multiplying phosphorus by 5

## DISCUSSION

The emulsifying agent is a prime factor in determining the tolerance of an animal for an emulsion<sup>10</sup>. Furthermore the stability of an emulsion depends on the emulsifying agent used. All emulsifying agents or stabilizers (surface active agents) are likely to be toxic to a certain extent. Consequently it is important to reduce the emulsifying agents to the minimal amount that will produce a stable fat emulsion. In the present study the amount of the combined emulsifying agents has been reduced to a minimum. Dextrose was added for its osmotic effect and as an aid in making the emulsion more stable.

Egg lecithin has been used as an emulsifying agent in preparing fat emulsions by most investigators.<sup>1-6a</sup> Dunham and Brunschwig<sup>1</sup> also used "demul 14" a mixture of polyglycerolesters to fulfill this purpose. McKibbin and associates<sup>10a, 10c</sup> found purified soya phosphatides (Asolectin) to be the most satisfactory emulsifying agent. The toxic effects of fat emulsions used in previous studies have been discussed recently.<sup>10b, d</sup> According to the report of Ashby<sup>1</sup> the purity of lecithin may be a factor in causing those reactions.

In the early part of the present study Tween 20 and Span 20 were used as emulsifying agents. The injection of these emulsifying agents produced toxic

manifestations which were mainly due to Tween 20. The toxic manifestations included dilatation of blood vessels, fall in blood pressure, urticaria, urination, defecation, and vomiting. The mechanism of the production of this group of reactions is not well understood. It is probable that these reactions are at least partly due to histamine formation in the body, for they can be prevented by subcutaneous injection of epinephrine five to ten minutes prior to the injection of Tween 20. Benadryl was also partially effective in preventing these reactions. Toxicity of the Tweens has also been shown by animal feeding tests by Krantz.<sup>1</sup>

The Spans can be ingested in comparably large quantities, possessing a nutritional value somewhat less than that of neutral fat.<sup>23, 26</sup> Injection of 0.5 per cent Span 20 in 10 per cent dextrose solution at a rate of 15 ml per minute in a dog weighing 12 kilograms produced no toxic effect, and no hematologic changes were demonstrated. The emulsifying agents used were Span 20, Asolectin (purified soya phosphatides), and sodium cholate. The injection of the emulsifying agents or of the fat emulsion stabilized by the emulsifying agents produced no fatalities and no apparent toxic effects under the conditions of these experiments. The appetites of these experimental animals were unaffected by the injections.

Diminution in blood pressure in experimental animals following intravenous injection of bile salts has been demonstrated by many investigators.<sup>27</sup> The sodium cholate present in the emulsifying agents and fat emulsion failed to cause any such change. Obviously the fall of blood pressure following bile salts injection depends on the amount and concentration given and on the rate of injection.<sup>28</sup> Fat emulsion injected at 1.5 cc per minute or 0.125 cc per kilogram per minute caused no fall of blood pressure. This is due to the fact that the injection was given slowly with a comparatively low concentration of sodium cholate. The sodium cholate given was probably eliminated from the circulation in a short period of time.<sup>27, 29</sup>

The cause of the increase of bile flow was probably the presence of sodium cholate since the choleretic effect of bile salts is well known.<sup>2, 29</sup> There was little change in urine flow, although one might expect some diuretic effect even with this amount of fluid or dextrose. Nevertheless it is important to point out that injection of fat emulsion or of emulsifying agents demonstrated no harmful effect on the mechanism of urine secretion.

The increase of lymph flow in the various groups of experimental animals was probably due to the hypertonic sugar solution in which the fat was contained. Even 0.9 per cent saline by vein or water by mouth increased the thoracic duct lymph flow as shown by Watkins and Fulton<sup>30</sup> and Ciandall and associates.<sup>1</sup>

The increase of total lipid output and lipid concentration of thoracic duct lymph may simply be due to the lymphagogue effect of these solutions as already discussed or to transportation of some of the injected fat to the lymph or to both. Reinhardt and co-workers<sup>32</sup> recovered 9 to 20 per cent of the intravenous radiophospholipids in the thoracic duct lymph after three to six hours. The mechanism and route of this transportation are obscure. It might be transported directly by way of the blood capillaries → tissue spaces → lymph capillaries and

to the lymph, or indirectly by way of the intestine where it would be hydrolyzed reabsorbed, resynthesized, passed into the lacteals and would finally reach the thoracic duct. However, the intestinal lacteals never appeared milky in six dogs during injection of fat emulsion which is evidence against the indirect way of transportation.

Some of the immediate toxic effects were salivation, pallor of tongue and mucous membranes, vomiting, and defecation, usually produced during the injection of fat emulsion and of emulsifying agents if the injection was given beyond 1.5 ml per minute, especially in those animals receiving the first injection. On the other hand, some of the animals would not show this group of reactions even if the injection rate was 2 to 2.5 ml per minute. Obviously there is individual difference in tolerating the fat emulsion or emulsifying agents. The mechanism responsible for producing these reactions is not well understood. Nevertheless the reactions can be prevented if the rate of injection is carefully controlled.

Secondary anemia was produced following the intravenous injection of fat emulsion in the experimental dogs of Dunham and Brunschwig,<sup>5</sup> McKibbin and associates,<sup>6b, 4c</sup> and our previous studies.<sup>7</sup> No investigators have discussed the cause of such anemia following fat emulsion injection in detail. Johnson and co-workers<sup>33</sup> and others<sup>34</sup> reported the destruction of red blood cells after fat ingestion. In their experiments they found that the lymph of the thoracic duct of dogs fed fat was markedly hemolytic. They further showed in animals that the daily excretion of the degradation products of hemoglobin was greater on a high fat diet than on one low in fat. The results of Johnson and co-workers<sup>33</sup> led Dunham and Brunschwig<sup>5</sup> to conclude that the production of secondary anemia in their fat infused dogs was similar to the anemia of the dogs fed a high fat diet.

In the present study, as Tables VII and VIII showed, mild anemia was produced in Dogs 4A and 4B, the animals injected with emulsifying agents and Dog 9, one animal injected with fat. These results may be explained by several possibilities.

(1) Lecithin itself (present in the purified soya phosphatides used as one of the three emulsifying agents) or its derivative might be the agent causing hemolysis. According to Lee and Tsai,<sup>3</sup> approximately 17 mg per cent of lecithin in physiologic saline solution is strongly hemolytic. In the present study, 120 mg per kilogram of soya phosphatides were given with each injection. The relatively slight increase in the lipid phosphorus of the plasma during or following injection indicates that the injected phosphatides are rapidly removed from the circulation.

(2) Sodium cholate used as one of the three emulsifying agents might be partially responsible for the anemia observed in three animals since it is well known that bile salts are hemolytic agents.<sup>27, 28</sup> The minimum concentration of sodium cholate in plasma necessary to cause hemolysis both in vivo and in vitro is not available. The amount of sodium cholate given (31.5 mg per kilogram) probably would not build up a concentration in the blood stream sufficient to be hemolytic.

(3) Infection was probably the main if not the only cause of anemia in the dogs infused with emulsifying agent (Dogs 4A and 4B), since the reduction of hemoglobin and red cell counts and the elevation of white cell counts were coincident with the application of plastic tubing which caused phlebitis at the site of injection

The accumulation of fat in the liver observed in Dog 7 following fat injection was not observed in other experimental animals, including both those given emulsifying agents and those receiving fat. The extent to which the liver contributes to the removal of intravenously injected fat remains to be studied, but it is reasonable to suppose that this organ takes up injected fat because of its role in fat metabolism and as the site of reticulo-endothelial tissue. In Dog 7 the serum phosphatase and dye clearance were not altered, and there was no sugar or albumin detected in the urine throughout the four week injection period. There was no evidence of impaired liver function other than the moderate increase in stainable fat, which suggests some delay in metabolism or transport. Probably impaired liver function should be a contraindication to the intravenous injection of fat.

The gain in body weight of Dogs 7, 8, and 9 during the period of fat injection was approximately the same as during the control period, when the same number of calories was ingested. Such experiments furnish no direct proof of fat utilization. The number of calories fed was in excess of that required for maintenance inasmuch as a weight gain occurred. This increase was probably due to fat deposition since there was no evidence of edema. The injected fat was not excreted in the urine or feces. According to the experimental evidences presented by McKibbin and associates,<sup>66</sup> injected coconut oil was metabolized in the body rather than stored, as judged by the iodine and saponification numbers of depot fat.

The protein-sparing action of fat is in dispute. Forbes and Swift<sup>67</sup> found that protein could be spared by adding lipid to the complete basal diet fed to their albino rats. The evidences of protein sparing action of fat are increased heat production, CO<sub>2</sub> production, O<sub>2</sub> utilization, and decreased urinary nitrogen output. On the other hand, Rappoport<sup>37</sup> states that the protein sparing action of carbohydrate is twice as great as that of an isodynamic quantity of fat. The output of creatinine falls below the starving level on a carbohydrate diet, but rises above it on a fat diet. Thus the nitrogen balance alone may not be very dependable in judging the utilization of the injected fat. However, the nitrogen balance during the infusion period was apparently the same as that for the control period while fat was given by mouth. The similarity of data for periods of ingestion and injection suggests but does not prove that the infused fat is utilized as well as that ingested.

The increase of serum lipids following fat injection is as one might expect. The relative increase of the total fatty acids, phospholipids, and total cholesterol was largely due to the relative amount of the individual substance contained in the fat emulsion. According to analysis, the fat emulsion contained 10.06 per cent of total fatty acids, 0.475 per cent of phospholipids, and 0.028 per cent of total cholesterol.

The complete disappearance of the injected fat from the blood stream is demonstrated in this experiment, took not more than seventeen hours and probably required considerably less time as judged by the decrease that occurred during the first four hours after injection. For example the serum lipids of Dog 4A were not far from the control level four hours after injection. These results are similar to the previous reports in regard to the disappearance of the lipemia following intravenous fat injection.<sup>2, 3, 38</sup> Little and associates<sup>39</sup> injected a patient intravenously with 1000 ml of chyle which contained 31 (m per cent of total lipids. The chylous fat disappeared from the blood circulation rapidly as indicated by the plasma lipids determination.

#### SUMMARY

Stable fine fat emulsions were prepared using Span 20 (0.5 per cent), Asolectin (0.4 per cent), and sodium cholate (0.1 per cent) as emulsifying agents homogenized with refined butter oil (10 per cent) by means of a high pressure Viscolizer.

Fat emulsions (in five dogs) and emulsifying agents alone (in three dogs) caused no fall in blood pressure. In the same animals urine and bile secretions were increased.

The thoracic duct lymph flow increased following injection of fat emulsion emulsifying agents, and 10 per cent dextrose solution. The total output of lymph lipids was increased following injection of fat emulsion but not following the emulsifying agents.

The daily intravenous infusion of emulsifying agents to dogs for four weeks failed to reveal toxic effects.

The daily intravenous infusion of 10 per cent butter oil emulsions to dogs fed a fat free diet resulted in further gain in weight and positive nitrogen balance similar to that of the control period.

Serum total fatty acids were markedly increased following fat injection. The increase of serum total cholesterol and phospholipids was comparatively slight. The serum lipids disappeared from the blood stream within seventeen hours after injection. No appreciable increase of serum lipids resulted following infusion of emulsifying agents alone.

Histologic examinations of the liver and kidney of dogs infused with the emulsifying agents showed normal structures. Of two fat infused dogs one showed essentially normal lungs, liver and kidney while the other showed fat granules in the liver cells.

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## HEMOPHILIA CURRENT THEORIES AND SUCCESSFUL MEDICAL MANAGEMENT IN TRAUMATIC AND SURGICAL CRISES\*

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IN REVIEWING the therapeutic management of forty-three patients with hemophilia observed over the past seventeen years in this Clinic, three more or less distinct periods can be recognized (1) The first was characterized by the use of a succession of nonspecific measures most of which were aimed toward increasing the available circulating thromboplastin. These included snake venom, ovarian hormones, placental extracts, and foreign protein sensitization, with horse serum or egg albumin as antigens. (2) Despite the use of these measures the continuing necessity for supportive, replacement blood transfusions during acute hemophilic bleeding episodes finally led to the recognition that whole fresh blood produced the most effective, even though transitory, reduction in the prolonged coagulation time of any measure then advocated. That fresh or frozen normal human plasma are just as effective as whole fresh blood was demonstrated shortly thereafter. (3) World War II gave a tremendous impetus to the chemical partition, isolation, and therapeutic study of particular fractions from human and bovine blood plasma. As a by-product of these important researches has come a so called antihemophilic fraction of human plasma ushering in a third era which is bringing new hope and optimism not only for the better clinical management of hemophilia, but also for the ultimate solution of the still enigmatic problem of the pathologic physiology and precise biochemistry of this dramatic familial disease entity.

Many theories have been evolved in the attempt to explain the abnormal coagulation mechanism of hemophilia. Most investigators agree that the primary impediment is in the retardation of the conversion time of prothrombin to thrombin. Calcium and a thromboplastic substance are necessary for this reaction. In analyzing the prevailing concepts, common accord is attained only in the fact that there is a lack of available circulating thromboplastin. The divergence of opinion is in the source and/or regulation of this catalytic or stoichiometric agent. Many substances have been demonstrated to have thromboplastic action. Extracts of nearly all animal organs—brain, lung, thymus, testis, muscle, and diffuse connective tissue<sup>1</sup>—show varying degrees of this activity. The ubiquity of tissue thromboplastin can be readily demonstrated in determining the clotting time in the hemophilic person. If a clean venepuncture is not obtained, allowing

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\*The plasma fraction I of Cohn used in this study was prepared from blood cells of from voluntary donors by the American Red Cross. This is one of a series of investigations in hemophilia being carried out with material supplied by the American National Red Cross. As soon as sufficient data become available to justify final conclusions concerning its therapeutic value a full report to the medical profession on the use of this plasma fraction in medical practice will be published.



only minute amounts of tissue juice to admix with venous blood a false lowering of the coagulation reading results. Proteolytic enzymes, Russell viper venom, saliva,<sup>2</sup> and, recently, urinary extracts<sup>4</sup> penicillin, streptomycin<sup>5</sup> and the products of roentgen radiation<sup>6</sup> have been shown to function directly or indirectly as thromboplastic agents.

Lysed platelets have long been known as one of the most potent sources of thromboplastin.<sup>1</sup> Therefore interest for many years has been focused on the possible functional abnormality or inadequacy of hemophilic platelets. Howell and Cekada<sup>7</sup> supported this concept, citing the observation that platelets from the hemophilic person fail to agglutinate readily *in vitro* and appear to resist prompt disintegration, thereby delaying presumably the liberation of their known thromboplastic content with a resultant prolongation of blood coagulation. Brinkhous<sup>8</sup> has recently presented data suggesting that a plasma factor is involved in the release of thromboplastin from platelets a factor deficient in the hemophilic patient. Quick,<sup>9</sup> however, indicates that the hemophilic defect may be due to the lack of a thromboplastin precursor which is quantitatively adequate in normal blood and is activated to thromboplastin by an enzyme from lysed platelets.

Ferguson hypothesizes that hemophilia is due to a deficiency in available trypsin, which according to his enzyme theory of coagulation is necessary for the conversion of prothrombin to thrombin.<sup>10</sup> It has been adequately demonstrated that trypsin both *in vivo* and *in vitro* will shorten the clotting time of hemophilic blood, albeit *in vivo* it is a rather hazardous procedure.<sup>11</sup>

As the cause of delayed blood coagulation in the hemophilic person and in contrast to the theory of thromboplastin deficiency Tocantins<sup>1</sup> <sup>12</sup> ascribes this phenomenon to an excess of circulating anticoagulant, so called anticephalin an antithromboplastic substance. In support of these conclusions he has accumulated a series of well controlled experiments.

Another of the hypotheses based on the lack of an essential circulating coagulation factor has evolved from the well known fact already stated, that the administration of fresh whole blood or plasma from a normal person will shorten the coagulation time in the hemophilic person whereas plasma or blood from another hemophilic patient is ineffective. Studies over the past eleven years at the Thorndyke Memorial Laboratory have resulted in the separation of a globulin from normal plasma capable of accelerating clot formation in the hemophilic subject.<sup>14</sup> <sup>21</sup> Under the direction of Dr. Edwin J. Cohn\* and as part of the war research plasma fractionation program, whole human plasma was separated on a physicochemical basis into five major portions.<sup>22</sup> The antihemophilic property was found to be most potent in fraction I which also contains fibrinogen. Fraction I separated from hemophilic plasma has been shown to be lacking in this specific activity.<sup>19</sup> This antihemophilic fraction now has been prepared from surplus pooled blood plasma in sufficient quantities for clinical evaluation and reports of the therapeutic effectiveness of this fraction are beginning to accumulate.<sup>17</sup> <sup>23</sup>

\*Of the Department of Physical Chemistry Harvard Medical School Boston Mass.  
†From the American National Red Cross

## CLINICAL STUDIES

Upon the demobilization of the Civilian Defense Program a supply of frozen human plasma was placed at our disposal for investigative purposes. These non-pooled units of 250 cc each were prepared by the prompt separation of all cellular elements, followed by rapid freezing of the whole plasma within a few hours after blood withdrawal from the donor according to the technique of Strumia, McGraw, and Reichel<sup>24</sup>. In the frozen state the antihemophilic activity of normal human plasma remains potent indefinitely and is readily available on rapid thawing in a water bath at 37° C, thus providing a convenient and effective therapeutic supply of the antihemophilic principle. Lyophilized plasma prepared within a few hours after withdrawal from the donor has been reported to be as effective as the original plasma<sup>25</sup>. This activity of thawed plasma, however, diminishes rapidly after several days' storage at 4° C. This loss of antihemophilic activity during storage of plasma in the liquid state has been reported by others<sup>26, 27</sup>. Similar rapid diminution of the antihemophilic principle has been observed *in vivo*, the activity lasting a maximum of not more than seventy-two hours, irrespective of the amount given. The duration of activity above certain amounts appears to be exponential<sup>28</sup>. (This of course varies with the degree of the coagulation defect in the individual patient. On the basis of repeated observations in severe hemophilic subjects, the maximum duration of effect usually can be obtained with 50 to 100 cc of fresh plasma or restored frozen plasma.)

Studies with the concentrated antihemophilic fraction of Cohn were initiated in this Clinic early in 1946 when material for clinical studies was made available\*. Certain selected cases from our clinical studies are presented to illustrate (1) the comparative effectiveness of the antihemophilic fraction I of Cohn versus freshly thawed plasma in promoting clot formation and (2) the possibility of successful medical management of the hemophilic patient during both elective and emergency surgery.

### *The Antihemophilic Activity of Plasma Fraction I —*

CASE 1—(Fig 1) At the time when the first supplies of plasma fraction I of Cohn were received, we were studying the effect of varying amounts of freshly thawed frozen plasma on a 17 year old hemophilic subject (Patient L. B.) who had been admitted for treatment of a large ulcerating lesion on the posterior surface of the right thigh, caused by the sloughing of a massive spontaneous intramuscular hemorrhage several months before. The comparative studies of plasma fraction I and freshly thawed frozen whole plasma are graphed in Fig 1.

The intravenous administration of 50 cc of freshly thawed (at 37°) frozen plasma brought an immediate fall in the coagulation time to normal values, which were maintained for twenty-four hours. Sixty hours later the coagulation time had returned to the prolonged base line values. Two units of fraction I (equivalent to 0.4 Gm of prothrombin dissolved in 10 cc of distilled water, were injected intravenously over a five minute period. This reduced the coagulation time to normal but held for less than four hours. Four units administered intravenously approximated the activity of 50 cc of thawed whole plasma.

\*Through the courtesy of Dr. Louis Diamond and later through the Medical Administration Committee, American National Red Cross.

†Coagulation times were determined in the following manner: 1 cc of venous blood were aspirated into a dry syringe, noting the time the blood first entered the syringe. The cubic centimeter was then ejected into each of three dry test tubes with an 8 mm. internal diameter; the fourth cubic centimeter was discarded. The first and second tubes were examined at intervals for clot formation. Coagulation of the third tube was accepted as the end point. (Normal 10 to 30 minutes.)

It is of some interest that, in a number of instances in which a study was made of this type of single injection titration, the coagulation time rebounded to higher than the original base line levels, producing a biphasic curve

CASE 2—(Fig 2) M S, a 5 year old boy, had received freshly thawed frozen plasma and whole blood at frequent intervals over two and one year years for the control

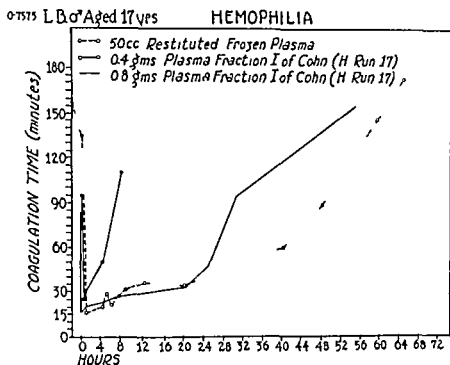


Fig. 1.—Case 1 Comparative effectiveness of plasma fraction I of Cohn against restored frozen plasma in the control of the coagulation defect in hemophilia

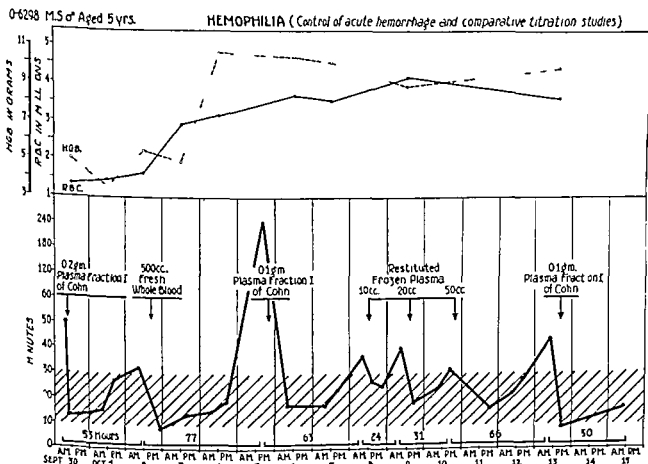


Fig. 2.—Case 2 The rapidly progressing hemorrhage into the subcutaneous tissues of the neck was checked by the control of the prolonged coagulation time with intravenous plasma fraction I of Cohn. Two days later transfusion of fresh whole blood alleviated the anemia as well as continued the maintenance of a normal coagulation time. Note comparative effectiveness of 0.1 Gm. of plasma fraction I of Cohn 10 cc and 50 c.c. of restored frozen plasma resulting in control of the coagulation time for sixty three twenty four thirty-one and sixty six hours respectively. The duration of effective control of the coagulation time with quantities of restored frozen plasma greater than 50 c.c. is apparently exponential.

of recurrent spontaneous hemorrhages. A maternal first cousin and great uncle were known hemophilic patients. This admission, the ninth, was precipitated by injuries to the right ankle and the left posterior cervical region resulting in the marked subcutaneous ecchymoses which had been progressive over the three days preceding hospitalization. There was a marked hematoma involving the entire left side of the neck, measuring about 13 cm. in diameter and extending from the surface plane of the neck approximately 8 cm, resulting in a fixation of the neck and head to the right. Ecchymoses were scattered over the entire body and extremities.

The initial coagulation time was 50 minutes. Cohn's fraction I, 0.2 Gm intravenously, brought about an immediate lowering of the coagulation time to 13 minutes. To ameliorate the severe posthemorrhagic anemia, the patient was given a transfusion of 500 cc of fresh whole blood, thus lowering the coagulation time from 32 to 8 minutes, normal coagulation being maintained for over sixty five hours. A rebound to over four hours was corrected with 0.1 Gm of Cohn's fraction I. Additional titration studies with increasing amounts of freshly thawed frozen plasma continued to maintain the coagulation time within the range of normality. Clinically there was clearing of the cervical hematoma to the extent that at the time of discharge there was only a small resolving surface ecchymotic area remaining.

The release of an increased quantity of antihemophilic globulin\* is allowing currently a more extensive clinical evaluation in a number of clinics. The excellent responses obtained during acute hemorrhagic crises in this as well as other clinics is encouraging.

*Surgery and the Hemophilic Patient*—Only in the last few years has even minor surgery in the hemophilic patient been undertaken without fear and trepidation. With a clearer understanding of the coagulation defect and with materials available for control, it is now possible to accomplish successfully major surgical procedures. Two illustrative case studies are presented.

CASE 3—(Figs 3 and 4) A. H., a 24 year old Mennonite farmer, walked into the emergency room of University Hospital apparently uninjured superficially following an automobile accident. Two companions appeared to be more seriously injured and immediate attention was shown them. The attending surgeon (V. A. D.), however, noticed the sudden development of diaphoresis and pallor in the first man and upon further questioning elicited complaints of increasing weakness and pain in the upper left abdominal quadrant. Examination revealed a small, superficial, bruised area at the costal margin, a rigid abdomen, and a state of imminent constitutional shock. The patient then informed the surgeon that he and other male members of his family had been under our observation for several years for hemophilia. A tentative diagnosis of traumatic rupture of the spleen was made, plasma transfusions were started immediately and simultaneously in both arms. Emergency surgical exploration revealed an abdomen full of free blood, its source being a small laceration at the inferior pole on the posterior surface of the spleen. Splenectomy was successfully accomplished and during the first twenty five hours the patient received 14 units of plasma (3,500 cc) and 4 units of whole blood (2,000 cc). On the second hospital day the coagulation time was 6 minutes. By the third hospital day, despite the tremendous quantities of borrowed blood and plasma, it had increased to 50 minutes, and oozing about the incision resulted in the loss of approximately 500 cc of blood. Thereafter daily transfusions of whole blood and/or fresh plasma were given, administered so as to maintain the coagulation index at all times under 30 minutes. Control of the hemophilic bleeding during the remaining period of convalescence was satisfactory and no further complications developed.

The genealogic history (Fig 4) revealed that both maternal and paternal grandfathers of the patient had been hemophilic. On the maternal side the patient had ten

\*By the Blood and Blood Substitutes Committee of the American National Red Cross.

## HEMOPHILIA

0-6929 Adm<sup>d</sup> Aged 24 yrs

### Emergency splenectomy for posttraumatic rupture

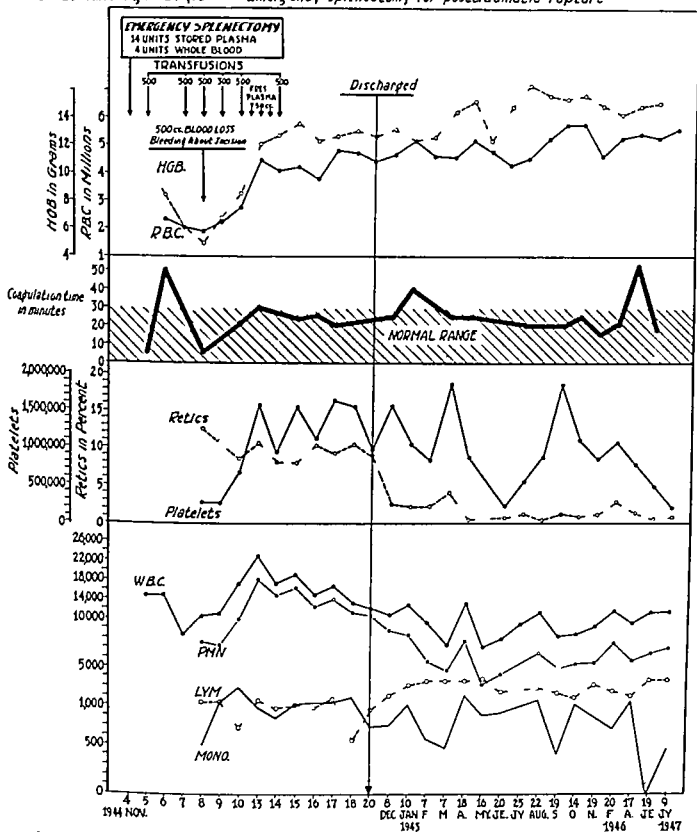


Fig 3—Case 3. The initial shock was corrected with 14 units of fresh liquid bank plasma and 4 units of whole fresh blood given during the first twenty four hours. Fifty six hours later there was an estimated 500 c.c. blood loss around the incision at which time the coagulation time was 50 minutes. Whole blood and freshly prepared plasma controlled the coagulation time during convalescence. Periodic follow up studies for nearly three years showed variations in coagulation time from 15 to 30 minutes. The genealogic history is diagrammed in Fig 4.

first cousins with known hemophilia five of whom had died from fatal hemorrhage secondary to trauma appendectomy, tonsillectomy leg ulcer and tooth extraction respectively. The patients

The patient was seen at two to three month intervals after the splenectomy thirty eight months ago and showed a vacillating coagulation time of from 15 to 55 minutes. He experienced no further clinical difficulty referable to the hemophilia and at the time of writing was in excellent health.

## HEMOPHILIA Genealogic History

O-6929 A.H. ♂ Aged 24 yrs

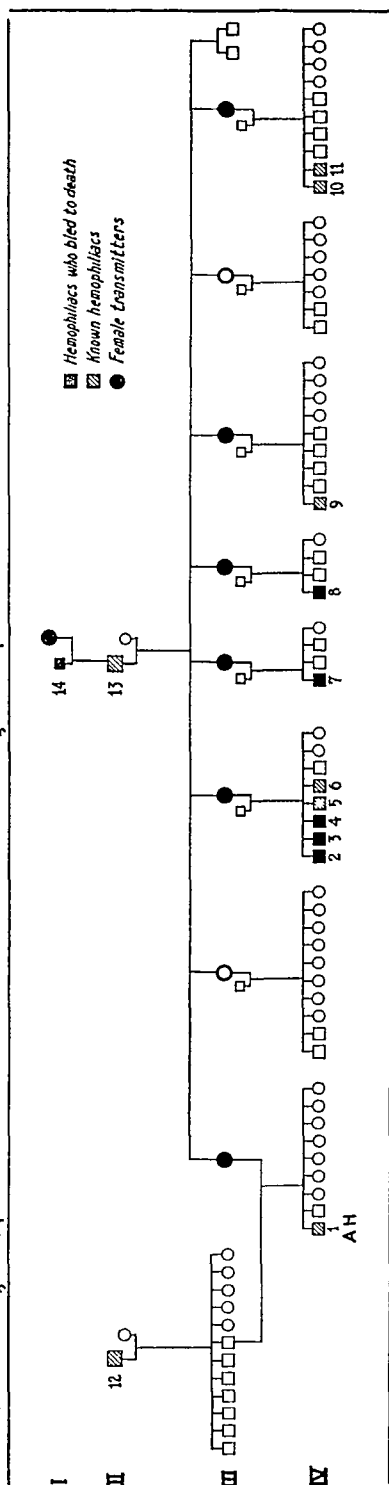


Fig. 4.—Genealogic history of Patient A. H. (Case 3) 1, whose hematologic data are charted in Fig. 3. Five of ten hemophilic cousins died from complications of hemophilia. 2 died nine days post appendectomy age 27, 8 died from internal hemorrhage following injury while loading wheat age 23, 4, died following hemorrhage from leg ulcer age 23, 5, a moderate bleeder age 43, was studied in this clinic and coagulation times were controlled with restored frozen plasma for tooth extractions age 23, 6, a moderate bleeder age 7, died from hemorrhage following tooth extraction age 21, 8 died from hemorrhage following tonsillectomy age 20, 9 moderate bleeder age 28, 10, moderate bleeder appendectomy four years ago, severe postoperative bleeding controlled with fresh blood, 11, severe bleeder, 12 the paternal grandfather was a severe bleeder, 13 maternal grandfather was a moderate bleeder, 14 a maternal great grandfather died to death following slight trauma.

CASE 4—(Fig 5) G F, a 26 year old white man, had experienced severe alveolar pain for eight months preceding his first admission to University Hospital. The patient had consulted several dentists for extraction of the diseased teeth but had been refused attention due to a severe hemophilic diathesis which had resulted in recurrent crippling hemarthroses since his fifth year of age. Profuse bleeding followed a tooth extraction at the age of 8 years. The alveolar pain was so severe and constant that for four months the patient had required Dilaudid, 4 mg every four hours. The initial coagulation time on admission was 125 minutes. Coagulation studies were made over a forty eight hour period preliminary to dental surgery. Comparatively frequent doses of freshly thawed frozen plasma were necessary to bring and keep the coagulation time within or near the range of normality. Determinations of the coagulation time were made three times daily and whenever values were on the upward trend additional plasma was administered. On the third hospital day, after receiving 250 cc of plasma preoperatively, extensive dental surgery\* was accomplished. The extraction required removal of a moderate amount of osseous mandibular tissue. Practically no hemorrhage occurred during the operative procedure. Throughout convalescence only minimal oozing from the operative site, normal for the degree and extent of the surgery, was evident.

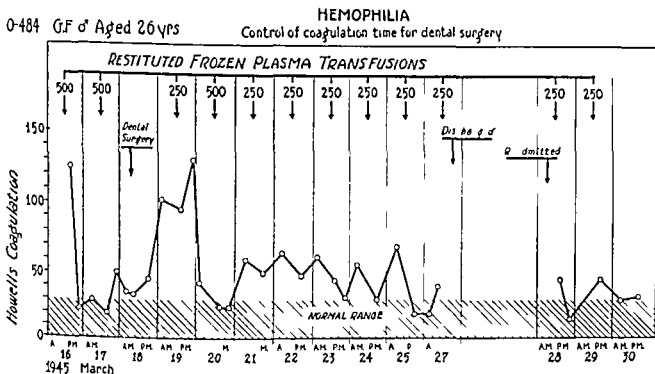


Fig 5.—Case 4. A forty eight hour control study preceding surgery indicated the expected response of the coagulation defect to restored frozen plasma. Extensive dental surgery was required to extract an impacted molar tooth. Operative and postoperative bleeding was minimal. Therapy continued for eight days. Two days after discharge trauma to granulating socket initiated hemorrhage requiring readmission. Coagulation time was 45 minutes. Additional plasma therapy controlled hemorrhage satisfactorily.

Rigid control of the coagulation mechanism was continued for six days postoperatively. Up to the time of discharge there was no hemorrhage. Two days later the patient inadvertently traumatized the oral granulating surface at the site of extraction with resultant profuse bleeding. Upon readmission the coagulation time was 45 minutes. Administration of freshly thawed frozen plasma again satisfactorily controlled the hemorrhage and convalescence was completed without further complications. The patient has since had another tooth extraction which was successfully managed under a similar regime.

#### DISCUSSION

Why does the hemophilic person bleed? Is it an inherent increased instability of the platelets? Is it an excess of an antithromboplastic substance? Is it a defect in trypsin, in a thromboplastic precursor, in antihemophilic globulin?

Most of the evidence in support of these hypotheses adds up to a *post hoc, ergo propter hoc* order of reasoning, and in examining one without consideration of the others, each is presented with convincing evidence and a good case is made. The ultimate solution may well represent a compromise between the presently held individual hypotheses.

The diagnosis of true hemophilia not infrequently presents a real problem. The classic criteria include (1) a familial history of the symptoms of hemophilia, (2) a personal history of more or less difficulty in controlling hemorrhage, especially following trauma, and (3) the demonstration of a prolonged coagulation time of venous blood. Recently, the demonstrable shortening of the coagulation time after parenteral administration of one of the antihemophilic globulins containing substances has proved to be a valuable confirmatory therapeutic test. Strange as it may seem, the differential diagnosis of hemophilia from other hemorrhagic diatheses, as for example thrombocytopenic purpura or hypoprothrombinemia, is not always immediately possible in an individual who is in a clinical remission at the time of examination. Vacillations in the clinical manifestations of either purpura or hemophilia reflect differences in the severity of the thrombocytopenia or the degree of prolongation of the coagulation time, respectively, from time to time (Fig. 3). A series of laboratory studies over a period of days, or even months, is occasionally necessary to establish a diagnosis. This type of study was necessary in a young physician who had experienced recurrent hemorrhagic episodes since infancy. A maternal uncle had been a bleeder. Both hemophilia and purpura had been clinically diagnosed at different times and in different clinics, though all previous coagulation studies had failed to show any abnormality and the platelets always had been found to be adequate in number. It was necessary to make serial daily studies of all phases of the coagulation mechanism. On only two of five consecutive days was a prolonged coagulation time found upon which to establish a hemophilic diathesis. Six months later an acute appendix was successfully removed under a regime of rigid coagulation time control. A similar study over a six month period was recently necessary in an 8-year-old youth to establish a recurrent thrombocytopenia as the definitive cause of hemorrhagic difficulties rather than a previously diagnosed hemophilia. *It is important that these fluctuations in the various factors of the coagulation mechanism from time to time be recognized, so that, if the specific abnormality or defect is not clearly defined and apparent on a single examination, repeated studies, particularly at the time of recurrence of symptoms, will be made.*

A rational approach to the successful therapeutic management of hemophilia must include three avenues: first, the use of specific therapeutic agents to promote coagulation of the blood per se, second, the institution of a prevention and guidance program through training the child with hemophilia in early life to adapt himself to and accept the various limitations enforced by the disease, and third, intelligent utilization of the physical therapist in overcoming the otherwise frequently crippling hemarthroses.

Control of acute hemorrhage in the hemophilic person is approached from two angles: prompt parenteral therapy in maintaining a normal coagulation



time for the duration of the emergency, and the local application of hemostatic agents where indicated and when possible

The active antihemophilic principle present in nonhemophilic blood is preserved only under certain conditions. The activity deteriorates rapidly under most *in vitro* conditions, therefore whole blood or plasma only when relatively fresh is effective. The coagulation promoting activity may be preserved by freezing or lyophilizing plasma within a few hours after withdrawal from the donor. Restitution of frozen plasma requires rapid thawing in a water bath at 37°, otherwise denaturation of the plasma proteins may occur with precipitate formation. Both properly prepared frozen plasma and lyophilized plasma provide excellent permanent, readily available sources of antihemophilic substance. The concentrated unit of powdered plasma fraction I of Cohn is reaquefied with distilled water and has a protein equivalent of 60 to 75 cc of plasma, with an antihemophilic activity of ten to fifteen times the comparable volume of plasma from which it came.<sup>9</sup> The fraction was prepared from blood which had been held as long as seventy-two hours before the separation of the cells and plasma had been accomplished. Greater potency would probably be obtained from fresh plasma preserved in the frozen state until fractionation. Intravenous or intramarrow administration has proved to be more reliable in reducing the coagulation time than administration by the intramuscular route.

The severity of the hemophilia governs the quantity and frequency of antihemophilic substance. If there is evidence of appreciable blood loss fresh whole blood will provide not only the antihemophilic substance but also replace the loss in red blood cells (see Fig 2). Fifty cubic centimeters of fresh, re-stored frozen or lyophilized plasma intravenously usually will maintain the coagulation time in a moderately severe hemophilic condition within safe limits for about twenty-four hours. One unit of Cohn's plasma fraction I (equivalent to 0.2 Gm protein) will accomplish the same for from three to sixty-five hours. In the hemophilic child the intravenous administration of these materials frequently presents a real problem when most of the available veins have been obliterated by numerous previous venoclyses. The intratibial or intrasternal routes have proved to be quite satisfactory in these instances.

A single venoclysis is generally all that is necessary for the immediate control of an acute hemorrhagic episode, for it is actually only necessary for the coagulation time to remain within normal values sufficiently long for a coagulum to form within the lumen of the hemorrhaging vessels. In the surgical patient it is advisable to have frequent determinations of the coagulation time preferably three times daily. In the presence of ascending values exceeding the upper limits of normal, further therapy for reducing the coagulation time is at once indicated (see Figs 3 and 5). Two to three days study of the relative ease or difficulty of control of the coagulation mechanism in the individual patient is desirable preliminary to elective surgery. Post-operative control is continued for from two to ten days depending on the severity of the hemophilia and the extent of the surgery.

In most instances parenteral control of the coagulation defect will suffice. However it is frequently desirable as an additional precautionary measure to employ local hemostatic agents at the operative site. Fibrin foam and thrombin have proved most effective. In the past, electrocauterization, gelatin, oxy cellulose, placental globulin, epinephrine, viper venom, muscle extracts, and globulin from human, bovine, rabbit and swine sources in addition to mechanical pressure aids have been used with varying degrees of success.

The rare development of a refractoriness in the lowering of the coagulation time by fresh whole blood, plasma, or the plasma fraction I of Cohn after repeated administrations has been observed.<sup>30-33</sup> This phenomenon has not been observed in this Clinic. Moderate fluctuations in the coagulation defect are the rule when individual cases are followed over long periods. Depending on the theory of the pathologic physiology to which one subscribes, this may be due to a vacillating quantity of antihemophilic globulin, anticephalin, thromboplastic precursor, or trypsin. It is conceivable that accentuations of these normal fluctuations may play an important role in the refractory states. The demonstration of an anticoagulant associated with the plasma globulin has been reported in several instances.<sup>31-33</sup> That the developing anticoagulant was an antibody and inhibited or tied up the antihemophilic globulin by means of an antigen antibody reaction has been proposed by Lawrence and Claddock.<sup>31</sup> Taylor<sup>30</sup> and associates have observed a refractory state develop in a patient who had previously responded to fresh whole blood, plasma, and the plasma fraction I of Cohn. Comparatively massive amounts of plasma fraction I of Cohn (10 Gm) and fresh plasma failed to effect significantly the prolonged coagulation time. Only when the greater part of the circulating blood had been replaced by fresh whole blood was normal coagulation again achieved. A similar massive replacement of the circulating blood volume is illustrated in our Case 3 (Fig 3).

The age range of the forty-three hemophilic patients at the time they were first seen in this Clinic is indicated in Table I. Fifteen of this group, or 34.8

TABLE I AGE RANGE OF HEMOPHILIC PATIENTS

AGE (YR)	NUMBER OF PATIENTS
3-5	10
5-10	5
10-20	9
20-30	12
30-40	6
>40	1
Total	43

per cent, were between 3 and 10 years of age. This age group presents the highest incidence of repeated hospitalizations for acute hemorrhagic episodes. The coagulation defect does not appear to be more severe at this age, but normal physical hyperactivity and an immaturity of perception make the child more subject to incidental and accidental trauma with initiation of hemorrhage. Many potential hazards can be overcome by the establishment of hemorrhage management program aimed toward the patient's understanding of and adaptation to his physical limitations. Through the close collaboration and cooperation of the

physician, medical social worker, parents and school authorities, it is usually possible to compromise on an outline of restricted activity which will allow the patient a relatively normal childhood while minimizing the frequency of acute hemorrhagic episodes and thus avoiding the invalid personality that is frequently seen in these patients. The severity of the disease, of course all too frequently dictates its own enforced limitations.

Eleven patients or 25.5 per cent of the group had experienced repeated hemarthroses which had left varying degrees of disability. The severity of the residua frequently totally incapacitated the patient. A program in conjunction with the physical therapist and orthopedist will often provide an opportunity to relieve an otherwise permanent disability. In a joint in which there has been fresh hemorrhage it is possible to aspirate the unclotted blood after control of the coagulation time has been effected with the parenteral use of antihemophilic agents. Physical therapy is administered in both the fresh and old arthroses only when and as the coagulation time is maintained within normal limits. The control of the coagulation time is maintained as outlined for the surgical patient.

#### CONCLUSIONS

Control of the prolonged coagulation time in the hemophilic patient may be effected by the intravenous or intramuscular administration of (1) fresh whole blood or plasma, (2) reconstituted frozen or lyophilized plasma that has been processed immediately after withdrawal from the donor or (3) the recently separated plasma fraction I of Cohn, a potent antihemophilic substance in our experience.

The temporary correction of the coagulation defect with any of these substances permits in the individual patient with hemophilia both emergency and elective surgical procedures with relative safety.

Physical therapy and carefully controlled orthopedic procedures may be successfully utilized in alleviating the frequently occurring hemarthroses when the coagulation time is maintained within normal values.

The institution of a guidance and prevention program through the cooperation of the physician, medical social worker, parents and school authorities will aid in assisting the hemophilic child to better adapt himself to the limitations enforced by his inherited disease.

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# EXPERIMENTAL STUDY OF THE COMPARATIVE ACTION OF HEPARIN AND DICUMAROL ON THE IN VIVO CLOT

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**I**N VIVO experiments concerning the action of heparin on the preformed venous clot have demonstrated its dual action of causing the dissolution of an early thrombus and of stimulating an adequate collateral vasculature by pass to a vein occluded by an organizing clot.<sup>1</sup> In view of the extensive use of dicumarol in venous thromboembolic disease, it was deemed advisable to investigate the action of dicumarol under similar experimental conditions. The purpose of this communication is to record the results of this comparative study and to summarize the literature pertinent to the subject.

## METHODS

Experimental venous thrombosis has been obtained by numerous chemical and mechanical methods,<sup>2-6</sup> most of which however are not so completely reliable as to allow for a conclusive statistical survey. Thus the injection of sodium ricinoleate<sup>6</sup> and crushing of the vein over an intraluminal silk thread<sup>6</sup> or stretching of the vein<sup>7</sup> have not proved satisfactory in our hands. The introduction of an extraneous factor in a chemically induced thrombosis complicates the proper evaluation of the anticoagulant to be tested so that we have resorted to a mechanical means of thrombus formation which produced consistent and predictable results.<sup>8</sup>

The genesis and subsequent elaboration of a thrombus depends on any or all of the following factors: stagnation of blood, injury to the intima, local release of relatively large amounts of thrombokinase.

A method utilizing these three factors has been reported elsewhere<sup>8</sup> and is as follows:

Adult rabbits weighing three kilograms are anesthetized with ether. The jugular veins are exposed and the most proximal portions securely ligated with silk. The distal portion of the vein is held over a narrow strip of metal and firmly struck twenty to thirty times with the handle of a scissors. In about two minutes after bleeding has ceased a palpable and visible clot is usually present. If clotting does not occur, the procedure is repeated. Thrombus formation invariably occurs with this procedure and the clot itself is indistinguishable from the in vivo clot seen in aseptic thrombophlebitis.<sup>8</sup>

Heparinization was effected by means of the heparin/Pitkin Menstruum preparation.<sup>9, 11\*</sup> The Pitkin menstruum is a gelatin base medium which was designed to regulate and retard the release of water soluble drugs incorporated within it. The preparation with varying amounts of heparin has been used extensively on human subjects with consistently satisfactory results. The formulas employed in our experiments contained vasoconstrictors which further delayed the absorption of the heparin and prolonged the effect of a single dose. The dosage has varied from 40 to 100 mg. of heparin given every two to three days. The amount was governed solely by the coagulation time which was maintained at not less than three to four times the normal level. The coagulation time was determined by the Lee-White-Howell method.<sup>12</sup> From previous studies we have found that rabbits react uniformly to the action of heparin.<sup>1</sup> To date we have encountered no heparin resistant rabbits.

Dicumarol was administered by both the oral and parenteral routes. We have found as has Link and associates, that rabbits vary in their response to oral dicumarol but that all

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rabbits respond to the intravenous administration of the disodium salt of dicumarol.<sup>13,14</sup> However, we have noted that, contrary to the observations of Link, rabbits orally dicumarol sensitive occasionally change in sensitivity so that the intravenous route becomes obligatory. Dicumarol was given orally in 6 mg doses. The disodium salt was prepared according to the procedure of Link<sup>14</sup> and was administered in 12 mg doses. The dosage and the interval between doses was governed essentially by the daily prothrombin time. Dicumarolized animals were fed a diet low in ascorbic acid which has been shown to be an antagonist of dicumarol.<sup>1</sup> In our early experiments, excessive doses of dicumarol caused loss of animals through coma, convulsions, and death. These toxic manifestations agree with the recorded data on the toxicity of this drug.<sup>16,19</sup> In no instance have we noted death due to hemorrhage, possibly because of a careful regard for the prothrombin time.

**Prothrombin Time**—Prothrombin time was determined on the dilute plasma by the Link modification<sup>13</sup> of Quick's method.<sup>20</sup> Fresh thromboplastin was used daily. Prothrombin times were recorded only when two successive determinations agreed within the arbitrary limits of 5 per cent. Sufficient dicumarol was given to maintain the prothrombin time above one and one half times normal, as measured in seconds.

**Scope of the Experiment**—Thrombosis was induced as described previously. On the ninth and fourteenth days after the induction of thrombosis, heparin or dicumarol was administered to alternate animals, respectively. Sufficient amounts of anticoagulant were given to maintain either the coagulation time or the prothrombin time well above clinically accepted limits. Anticoagulants were administered for two weeks. This arbitrary limit was set by our previous experiments<sup>1</sup> with heparin in which it was shown that the maximum effect was obtainable within that period. At the completion of this period the animals were anesthetized with ether, the veins were inspected, photographed in situ, and sections were taken for microscopic examination. There was no need to obtain control animals since our previous experiments<sup>1</sup> have yielded controls for all periods up to thirty days after the induction of thrombosis.<sup>1,5</sup>

## RESULTS

The scope and results of the experiment are seen in Tables I and II.

From Table I (anticoagulant therapy started nine days after induction of thrombosis) it may be seen that of the six veins heparinized for two weeks, four were patent (Fig 1) and two were occluded (Fig 2), while in a similar number of dicumarolized veins, two were patent (Fig 3) and four were occluded (Fig 4). All of the control veins were occluded. The visible collateral system appeared to be greater in the heparinized series.

TABLE I FOURTEEN DAYS OF ANTICOAGULATION THERAPY STARTED NINE DAYS AFTER INDUCTION OF THROMBOSIS

	NUMBER OF VEINS	PATENT	OCCLUDED	COLLATERALS
Heparin	6	4	2	++ to +++
Dicumarol	6	2	4	+ to ++
Controls	6	0	6	0 to +

TABLE II FOURTEEN DAYS OF ANTICOAGULANT THERAPY STARTED FOURTEEN DAYS AFTER INDUCTION OF THROMBOSIS

	NUMBER OF VEINS	PATENT	OCCLUDED	COLLATERALS
Heparin	8	6	2 Recanalized	+++ + to +++
Dicumarol	8	2 Patent 2 (Extensive recanalization)	4	
Controls	8	0	8	+

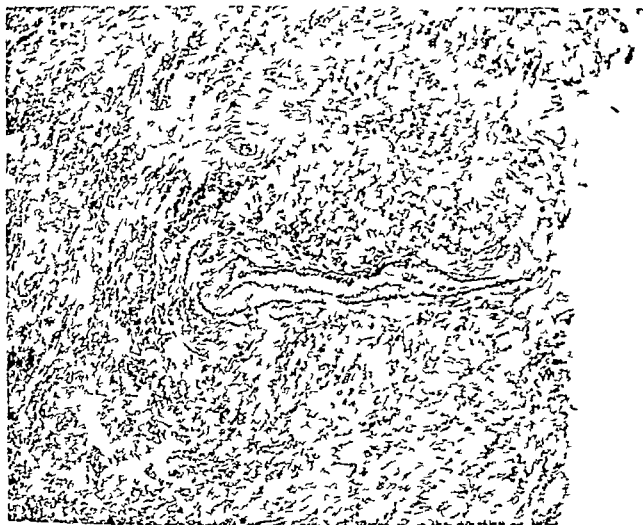


Fig 1.—Rabbit 40B nine days after thrombosis fourteen days of heparin. The lumen is narrow and empty. The internal elastic membrane is well defined. (Elastic van Gieson  $\times 80$ )



Fig 2.—Rabbit 61C nine days after thrombosis fourteen days of heparin. The lumen is almost completely filled with an organized thrombus. A portion of the original lumen remains. Note that this does not represent recanalization but the original lumen now formed in part by endothelial lined thrombus. (Elastic van Gieson  $\times 80$ )



Fig 3—Rabbit 63C nine days after thrombosis fourteen days of dicumarol. The lumen is patent. The elastica is fragmented. (Elastic van Gieson X80)



Fig 4—Rabbit 62 nine days after thrombosis fourteen days of dicumarol. The lumen is occluded by a thrombus in which areas of sludge formation are still present. Beginning organization is present at one margin. Despite the age of the clot (twenty three days) it presents all the characteristics of an early thrombus. (Elastic van Gieson X30)





Fig 5—Rabbit 31B fourteen days after thrombosis fourteen days of heparin. The lumen is patent. The internal elastic is reduplicated. (Elastic van Gieson  $\times 10$ )

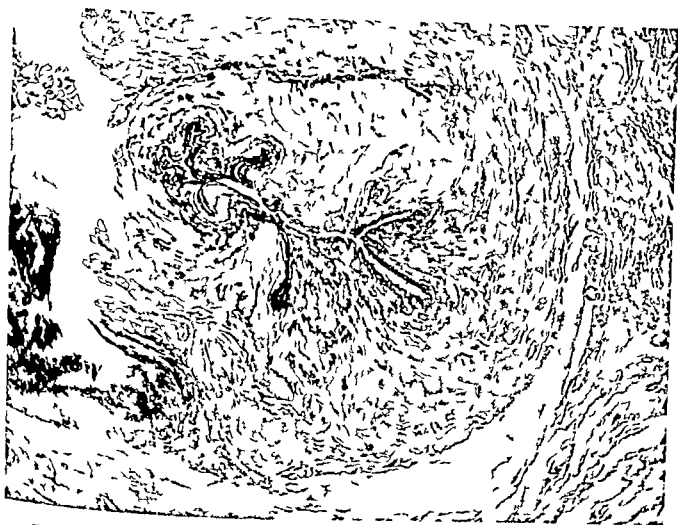


Fig 6—Rabbit 59C fourteen days after thrombosis fourteen days of dicumarol. The lumen is contracted but empty. The internal elastic is markedly fragmented. (Elastic van Gieson  $\times 10$ )

From Table II (anticoagulant therapy started fourteen days after induction of thrombosis) it is evident that of eight heparinized veins, six were patent microscopically (Fig 5) and two showed extensive recanalization. Of eight decannulized veins, two were patent (Fig 6), two showed extensive recanalization, while four were occluded. These last four were considered occluded although they demonstrated recanalization. The recanalization was, however, similar in extent to that of the control veins. The lumina of all the patent veins were markedly narrowed and showed considerable subintimal proliferation. All vessels appeared to be occluded on gross inspection, patency being established by microscopic section. The collaterals were far greater in size and number in the heparinized group.

#### DISCUSSION

The results which we have recorded represent the inevitable continuation of our investigations into the physiology of the anticoagulants. It would seem proper at this point to review the experimental work on this subject since such an historic survey reveals horizons not ordinarily perceptible to the worker in such a narrow field.

*Heparin*—With the discovery of heparin by McLennan<sup>1</sup> and its subsequent elaboration by Howell<sup>22</sup> the ability of this substance to retard coagulation stimulated many workers to find a suitable application in experimental work with an eye toward the therapeutic value in disorders of blood coagulation. Howell and McDonald<sup>23</sup> demonstrated the innocuousness of the purified material when injected intravenously in dogs and in man and showed at the same time that heparin had no effect on the number of circulating red cells, leucocytes, and platelets. Lack of toxic action of the purified material was demonstrated by Reed<sup>24</sup> in several hundred experiments.

The *in vivo* action of heparin differs from the *in vitro* action in which purified reagent is used. Thus, in a mixture of purified fibrinogen and thrombin, heparin has almost no inhibiting action and therefore shows no retarding or blocking effect on the conversion of prothrombin to thrombin.<sup>2</sup> Heparin is not ordinarily neutralized *in vitro* by thromboplastin.<sup>2</sup> The *in vivo* action of heparin appears to depend primarily on the combined action of heparin and an undesignated fraction of the serum albumins, the so called albumin X of Quick, in the presence of neutral salts.<sup>26</sup> The importance of this factor has been conclusively demonstrated.<sup>2</sup> Thus, heparin appears to be an antithrombogen, that is an agent which reacts with a constituent in the plasma to form a true antithrombin.<sup>28, 29</sup> The actual method whereby heparin interferes with the processes of coagulation depends on its very high negative charge. According to Torpes,<sup>30</sup> heparin, by virtue of its high sulfuric acid content, contains the strongest electric charge of any high molecular substance in the body. Apparently heparin exerts its action through this charge. This seems to be supported by the neutralizing effect of basic protamine, which has the property of promptly counteracting the action of heparin. The multiple *in vivo* effect of heparin on thromboplastin, prothrombin, and thrombin is more readily explained as a loading and unloading of negative charges on the proteins concerned.

The acknowledged ability of heparin to prevent the coagulation of whole blood by interfering with the formation of thrombin requires no further comment. It is the action on a pure platelet thrombus which appears to be of greater concern. The starting point of such a platelet thrombus is a small mass of agglutinated platelets. The prevention of this clumping is probably the primary action of heparin in the prevention of platelet thrombosis.<sup>31</sup> Although earlier *in vitro* investigations<sup>31</sup> revealed that crude heparin was not effective in preventing the agglutination of platelets, subsequent experiments have revealed that in blood to which heparin has been added there has been no clumping of platelets or diminution in their number.<sup>32-34</sup> The agglutination of platelets is presumed to be due to an adhesive agent and not to formed fibrin,<sup>45</sup> since several workers have been unable to demonstrate the precipitation of

fibrin in white thrombus formation.<sup>30</sup> A much higher concentration of heparin is required to prevent the agglutination of platelets than is required to inhibit the coagulation of blood.<sup>31</sup> Another way of stating this would be to say that although the adhesion of platelets is reduced in the presence of heparin it has been noted that more heparin is required to prevent the agglutination of platelets than to inhibit the formation of fibrin. In rabbit and human blood the platelets of which are either more readily killed or liberate a more powerful adhesive agent,<sup>32</sup> heparin retards but does not completely prevent platelet thrombus formation.<sup>33</sup> With sufficiently large doses of heparin the effect on platelet agglutination develops after a latent period unlike the immediate effect on the clotting time.

The effect of heparin on in vivo thrombi has been studied in three ways.

1 *Action on Thrombi in a Glass Cell or in Cellophane Tub.* Bet and co-workers<sup>34</sup> showed that the administration of a large dose of purified heparin prevent or delay the formation of white thrombi in glass cell or cellophane tube interposed between the carotid artery and the jugular vein. It has been similarly shown that when glass cannula are interposed between the cut ends of a large artery adequate heparinization prevents the occlusion of the cannula for periods greater than twenty-four hours while control cannula become occluded after twenty minutes of active blood flow.<sup>40</sup>

2 *Action on Thrombi in the Coronary Artery.* If the endocardium of the left ventricle of a dog was injured by the injection of sodium ricinolate and the myocardium injured by ligating the anterior descending branch of the left coronary artery large mural thrombi very quickly formed in the lumen of the left ventricle. When however adequate amount of heparin were given before the injury was produced mural thrombi were not seen.<sup>41</sup> Similarly it has been shown that coronary thrombi is could be induced by sodium ricinolate injected directly into the coronary vessels in twelve out of thirteen dogs while similar lesion occurred in only one of twelve dogs if heparin was given continuously for twenty-four hours after injury.<sup>41</sup>

3 *Action on Thrombi in Injured Veins.* Morris, Bet and co-workers<sup>42</sup> were able to cause intraluminal venous thrombosis by either crushing a vein over an intraluminal silk thread or by the injection of sodium ricinolate into the vein. In either case thrombosis occurred in 80 to 85 per cent of the control. Prophylactic heparinization preceding trauma with subsequent heparinization after trauma resulted in a few minimal thrombi. Heparin administered after trauma maintained the patency of a considerable number of veins. Thus 14 per cent of the control veins remained patent after mechanical trauma while 41 per cent of the test veins retained their patency if heparin was administered for seventy to seventy-two hours. Rabinovitch and Pines induced thrombosis by stretching the vein and then causing a partial obstruction to the blood flow by a constricting ligature. The experimental results showed that in certain instances heparin caused the disappearance of the thrombus only in the early stages and never when the clot already had been organized.

In an attempt to determine at what stage dissolution of the clot took place and what if any was the action of heparin on the organizing clot we<sup>43</sup> induced thrombosis in a manner described earlier in this paper.<sup>8</sup> Using heparin/Pitman menstruum which would yield a constant anticoagulant effect for at least forty-eight hours, it was found first that patency can be reestablished in a number of veins even as long as six days after a clinically palpable and microscopically acceptable thrombus is present. Second the extent and apparently the speed of recanalization is enhanced by the use of heparin. Third when the vein is so occluded as to preclude the resumption of clinical patency recanalization was still greater in degree and extent under heparin therapy and fourth in the presence of occluded veins which cause definite obstruction to circulation the opening of adjacent collateral venous channels is so extensive in the presence

of heparin that the combined cross-sectional area of the collateral system appears as great as, if not greater than, that of the original vein. We found, in addition, that in every instance of sludge formation such as has been described in experimental frostbite, *Plasmodium knowlesi* malaria and traumatic shock<sup>44, 45</sup> heparin caused complete solution of the clot with resumption of clinical patency.

**Dicumarol**—The discovery of the entity known as sweet clover disease by Schofield,<sup>46</sup> its further elaboration by Roderick,<sup>47</sup> and finally the magnificent researches of Link and associates<sup>13, 16, 21, 53</sup> led to the isolation and synthesis of dicumarol, the causative agent of this disease. The physiologic effects of dicumarol as measured by the assay of circulating prothrombin<sup>13, 20</sup> are too well known to require further elaboration. Animals are presumed not to acquire immunity or increased susceptibility to this hemorrhagic agent<sup>13</sup> although we have found this not to be absolutely true. However, animals which are insensitive to the oral administration of the drug usually respond to the intravenous administration of the disodium salt of dicumarol.<sup>14</sup> The administration of a single dose effects a reduction of the prothrombin level without producing gross signs of permanent injury. The immediate effects of a massive toxic dose are dyspnea, hyperthermia, vasodilation, convulsions, coma, and death. Since these effects occur within twelve hours, there is no reduction in prothrombin time and consequently no hemorrhage.<sup>1, 11</sup> The continued feeding of this substance is necessary for the production of hemorrhages.<sup>16</sup>

The action of dicumarol is not clearly understood. However, studies indicate that it prolongs the prothrombin time and hence the coagulation time in animals<sup>17, 54</sup> and in man.<sup>55</sup> It has been shown that the action of dicumarol influences both the production of fibrinogen and the synthesis of prothrombin.<sup>56</sup> The thirty-six to forty-eight hour lag in response<sup>13</sup> corresponds to the time necessary to utilize completely the prothrombin circulating in the blood. Vitamin K<sup>48, 49</sup> counteracts the anticoagulant effects of dicumarol while vitamin C probably intensifies this protective action of Vitamin K.<sup>50</sup> Dicumarol is ineffective *in vitro*.<sup>1</sup>

Platelet adhesiveness is undoubtedly a factor in thrombosis. Wright<sup>50</sup> observed an increased stickiness at the time when, statistically, thrombosis is likely to occur. There was a simultaneous increase in the platelet count. She thus postulated that the large numbers of newly formed platelets are hyperadhesive. The same observer noted that the greater the concentration of heparin the less is the stickiness of the platelets.<sup>58</sup> The adhesiveness of platelets also definitely decreases after dicumarol administration<sup>33, 61</sup> and bears a direct relationship to the prolongation of the prothrombin time. However, the prothrombin time must be decreased significantly before an actual decrease in platelet adhesiveness can be demonstrated.<sup>32, 62</sup> Dicumarol causes no appreciable difference in the number of circulating platelets.<sup>32, 34, 62, 63</sup> Clot retraction is retarded in the presence of sufficient amounts of dicumarol to elevate the prothrombin time significantly.<sup>56, 57</sup>

The experimental studies concerning the effect of dicumarol on the thrombus and thrombus formation have paralleled the heparin investigations. Experimental intravascular clotting ordinarily does not occur in animals which are under full dicumarol effect.<sup>64</sup> Glass cannulas interposed between two ends of an artery were seldom occluded when animal had previously received dicumarol. Patency was maintained for six to eight hours if the prothrombin time was elevated above thirty minutes in dogs.<sup>54</sup> In the glass cell, around artery-jugular vein anastomosis,<sup>57</sup> Dale and Jaques<sup>65</sup> have shown that dicumarol appears to be slightly more effective than single doses of heparin in the prevention of thrombosis. This work was predicated, however, on one dose of heparin, the effects of which are known to disappear in a few hours. Richards and Cortell,<sup>66</sup> using the ricinoleate method of thrombosis,<sup>66</sup> demonstrated the protective action of dicumarol in the three and six day experimental animal groups; in their series, the incidence of thrombus formation was much less than in the untreated controls. Others<sup>63</sup> have shown that the administration of dicumarol decreases considerably the tendency to thrombosis in veins which have been crushed over an intraluminal silk thread. When blood was trapped in portions of the jugular and femoral vein, delayed thrombosis was apparent in dicumarolized dogs. When thrombosis did occur, the clot was softer and more friable than in the control animals.<sup>54</sup>

## COMMENT

The experiment which is herein reported represents the logical step in the progressive series of attempts to elucidate the effect of the anticoagulants on the in vivo thrombus. Our knowledge thus far indicates that in the presence of heparin all clots undergo solution if they are in the sludge stage. This is not true of dicumarol because of the time lag between the administration of the drug and the effective prolongation of the prothrombin time. However beyond this initial stage both anticoagulants effectively caused resumption of clinical patency in a considerable number of veins which were occluded by clots for four days or longer, even up to two weeks in duration. This effect is at variance with the commonly accepted knowledge of thrombus behavior. We are at a loss to explain adequately the solution of a thrombus whose individual platelets appear to have lost their microscopic identity. However since it has been pointed out that the agglutination of platelets is due to the presence of an adhesive agent which is not formed fibrin,<sup>3</sup> and since some workers have been unable to demonstrate the precipitation of fibrin in white thrombus formation<sup>3a</sup> it may be presumed that possibly physical or physiochemical factors of which we are as yet unaware play a role. A possible lead in this direction may be derived from studies of the clot resistance in tails of dicumarolized mice.<sup>67</sup> In this experiment it was thought that the hemorrhagic condition induced by dicumarol might not be based solely on the prolonged coagulation and prothrombin times or on the decreased firmness of the clot but on the marked capillary dilatation. This widening of the vessels may contribute in a purely physical sense to the initially decreased clot resistance. Thus, small areas may break off because of the impact of an increased volume of blood and may lodge elsewhere in the body. Because of the lack of coagulability of the blood, these small emboli retain their minute size are unable to propagate and thus are rendered innocuous regardless of where they may lodge. On the other hand since clotting is essentially a physiochemical process which is theoretically reversible the preponderance of the equation factors directed against clotting, by virtue of the excessive amounts of emulating anticoagulant material (or its physiologic equivalent), may possibly render into solution a clot which has not yet become organized.

Concerning the relative efficacy of heparin and dicumarol we favor the former. Although this may be attributed to our extensive experience with heparin, we nevertheless have found that it is easier to work with (in the Pitkin menstruum), has more predictable anticoagulant levels is safer and requires a less elaborate laboratory check to maintain safe and effective anticoagulation responses. The action of heparin is more prompt as a result of which all clots undergo resolution if they are in the sludge stage. This is not always the case with dicumarol because of the time lag between administration of the drug and effective prolongation of prothrombin time. The degree of collateralization appears greater with heparin.

## SUMMARY

The mechanism of clot formation and the blocking action of the anticoagulants heparin and dicumarol are critically reviewed.

A comparative study was made of the properties and merits of heparin and dicumarol. The relative ability of these anticoagulants to (a) prevent clotting (b) resolve thrombi, and (c) promote an effective vascular by-pass was evaluated.

On the basis of this comparative study it would appear that heparin is superior to dicumarol.

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# THE EFFECT OF DIETHYLSTILBESTROL ON BLOOD LIPIDS AND THE DEVELOPMENT OF ATHEROSCLEROSIS IN CHICKENS ON A NORMAL AND LOW FAT DIET

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THE occurrence of large cyclic changes in the calcium phosphorus and lipids of the blood in egg laying vertebrates during periods of egg production is a well known phenomenon. The literature bearing on this subject has been amply reviewed by Riddle<sup>1</sup> and by Gardner and Pfeiffer.<sup>2</sup> The variations are considerable, amounting to a threefold increase in calcium<sup>22</sup> phosphorus<sup>4, 5</sup> and lipids<sup>6, 26, 28</sup> during the egg laying state. Lorentz, Entenman and Chaikoff<sup>6</sup> showed that the increase in the blood lipids consisted of changes in the neutral fat, phospholipid and free cholesterol fractions.

Riddle<sup>7</sup> was the first to suggest that the ovarian hormone was in all likelihood responsible for this increase in blood calcium phosphorus and lipids in the laying bird. With the advent of estrogenic substances it was found<sup>8, 9, 24</sup> that administration of these substances resulted in marked increases in the blood calcium phosphorus, and lipids duplicating the changes which occurred cyclically during the egg laying period. These changes can also be induced in the male of the species by the exhibition of estrogenic substances. The changes described have now been observed by many workers using natural and synthetic estrogens given both orally and parenterally.<sup>10, 13, 2</sup> Gonadotropins also cause an increase in the blood levels of calcium phosphorus and lipids.<sup>23</sup>

In 1946, Lindsay, Lorenz, Entenman and Chaikoff<sup>14</sup> reported that they had been able to produce hyperlipemia and atheromatosis in chickens by implanting pellets of diethylstilbestrol. They employed cockerels which were more than 3 months of age at the beginning of the experiment and sacrificed them after six to seven months so that their animals were approximately 9 to 10 months of age at the conclusion of the experiment.

Dauber<sup>1</sup> has shown that spontaneous atherosclerosis occurs in chickens after 5 months of age and becomes increasingly frequent with increasing age of the animals. While the lesions which she described were predominantly in the abdominal portion of the aorta and those described by Lindsay and co-workers<sup>14</sup> were predominantly in the thoracic aorta nevertheless the possibility exists that the results obtained by Lindsay and co-workers may have been due to spontaneous lesions arising because of the age of the chickens at the time they were sacrificed.

Therefore we have repeated the stilboestrol experiment using young birds 6 weeks of age at the beginning of the experiment which were sacrificed when

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the birds were approximately 6 to 7 months of age. Thus we attempted to obviate the possibility that the lesions observed were of the spontaneous variety described by Dauber. In addition we investigated the effect of stilboestrol on cockerels maintained on a diet very low in fat and cholesterol. During the course of this study we were able to make observations on the effect of a low fat diet on the blood cholesterol of the chicken and on the occurrence of the spontaneous variety of atherosclerosis in these animals. The latter observations are in the nature of a preliminary report, as the effect of a low fat diet on the occurrence of atherosclerosis is under long-term investigation in our laboratory at the present time.

#### PROCEDURE

Thirty six white Leghorn cockerels 6 weeks of age at the beginning of the experiment were used. Ten were maintained as controls and received chick starter mash and water ad libitum. An additional six birds were maintained on chick starter mash and water, and pellets of diethylstilbestrol,\* 25 mg each, were implanted subcutaneously at the beginning of the experiment and four and eight weeks later. The remaining twenty birds were maintained on a diet of chick starter mash from which fat and cholesterol had been extracted by a commercial degreasing process employing alcohol and ether. The fat content was reduced from 4.4 per cent to approximately 0.3 per cent†. The extracted mash was dry and powdery, and 8 per cent water was added. To replace the caloric value of the fat removed, 5 per cent sucrose was added<sup>16</sup>. To compensate for the destruction of vitamins in the process, supplements were added. The weekly supplements were as follows: vitamin A, 3,600 I.U., vitamin D, 40 A.O.A.C. units, vitamin E, 90 mg, vitamin B, 27 Gm. of brewers' yeast<sup>17</sup>. The fat soluble vitamins were given in very highly concentrated form in a few drops of cottonseed oil twice a week. The yeast, which contained 5.8 per cent of fat, was mixed with the feed once a week.

Diets of similar composition have been found not to retard the growth of chicks for periods of fourteen weeks<sup>16</sup>. Eleven of the twenty birds were maintained as controls and nine received implants of stilboestrol pellets. In three the pellets were not implanted until the animals had been on the low fat diet for almost one month. In the other six, the pellets were implanted at the beginning of the experiment and at four and eight weeks thereafter. Surviving animals were sacrificed when 25 to 30 weeks of age, or after nineteen to twenty four weeks on their respective diets. Hearts and aortas were carefully dissected out and examined grossly for the presence of atheromatous changes. Lesions were graded from 0 to 4 on an empirical basis for severity of atherosclerosis. Several of the aortas were sectioned and examined microscopically. Blood was drawn from the alar vein at three week intervals, and total cholesterol determinations were made by the method of Schoenheimer and Sperry.

\*The stilboestrol was generously supplied by Eli Lilly & Company, Indianapolis, Ind.

†We are indebted to The Armour Laboratories, Chicago, Ill. for degreasing large quantities of mash.

‡We are indebted to Lederle Laboratories, Inc., New York, N. Y. for our vitamin supply.

## RESULTS

*Atherosclerosis of the Aorta —*

*Normal Controls* Ten birds were maintained on chick starter mash and water for periods ranging from two to twenty five weeks. One died after two weeks, one after seventeen weeks, two after eighteen and one half weeks, four

TABLE I. ARTERIOSCLEROSIS IN CONTROL CHICKENS FED ORDINARY CHICK STARTER MASH AND WATER AD LIBITUM

WEEKS FED	AGE AT TIME OF DEATH (W.K.)	DEGREE OF ATHEROMA OF AORTA		LIVER PRESENCE OF EXCESS FAT
		THORACIC	ABDOMINAL	
2	8	0	0	0
17	26	0	0	0
18½	24½	0	1	0
23½	29½	½	1	0
18½	24½	0	1	0
25	35	0	0	0
19	25	0	0	0
19	25	0	0	0
19	25	0	1	0
19	25	0	0	0

after nineteen weeks and one each after twenty three and one half and twenty five weeks on the plain mash diet. Four of the birds showed gross atheromatous lesions of the aorta. One of the four had a minor lesion in the thoracic aorta (grade ¼), and all four had moderate lesions of the abdominal aorta (grade ½) of the type described by Diab<sup>13</sup> as typical for spontaneous atherosclerosis.

*Ordinary Mash Plus Stilboestrol Implants* Six birds were maintained on chick starter mash and had three 25 mg pellets of stilboestrol implanted at four week intervals. One animal died after twelve weeks on this diet; the heart and aorta were not examined. One bird died after nineteen weeks and the remaining four were sacrificed after twenty three weeks on this diet. The chicken which died after nineteen weeks of feeding had no lesions of the aorta. The remaining four birds had atheromatous lesions of varying degrees of severity. Three had lesions in both the thoracic and abdominal portions of the aorta

TABLE II. ARTERIOSCLEROSIS IN CHICKS FED ORDINARY CHICK STARTER MASH AND WATER AD LIBITUM AND IN WHICH 25 MG PELLETS OF STILBOESTROL WERE IMPLANTED AT THREE TO FOUR WEEK INTERVALS

WEEKS FED	AGE AT TIME OF DEATH (W.K.)	DEGREE OF ATHEROMA OF AORTA		LIVER PRESENCE OF EXCESS FAT
		THORACIC	ABDOMINAL	
12	18	—	—	F
19	25	0	0	0
23	29	½	0	0
24	29	—	1	0
23½	29½	2	—	½
24½	29½	1	1	0

F Fatty liver  
Aorta to 1

and one had lesions in the thoracic portion of the aorta only. The lesions were moderately severe grading from ½ to 2. A representative autopsy protocol on one bird is presented. Heart and aorta—the mitral and aortic valves were

TABLE III ARTERIOSCLEROSIS IN CONTROL CHICKS FED LOW FAT CHICK STARTER MASH SUPPLEMENTED WITH VITAMINS A, B, D, AND E

WEEKS FED	AGE AT TIME OF DEATH (WK)	DEGREE OF ATHEROMA OF AORTA		LIVER, PRESENCE OF EXCESS FAT
		THORACIC	ABDOMINAL	
11	17	0	0	0
18½	24½	0	0	0
18½	24½	0	0	0
23½	29½	0	0	0
23½	29½	0	0	0
3	9	0	0	0
19	25	0	0	S F
19	25	0	0	0
19	25	0	0	0
19	25	0	0	0
19	25	0	0	0

S F Slightly fatty liver

somewhat thickened and showed very fine pin-point yellow deposits in their substance. The brachiocephalic arteries and the thoracic aorta were thickened, and there were elevated yellow patches in both. There was a fine pin point yellow plaque running from the origin of the renal arteries to the bifurcation of the aorta.

*Low Fat Diet Controls* Eleven chickens were maintained on the low fat diet described. Of these, one died after three weeks and another after eleven weeks on this diet. The remaining birds survived from eighteen and one-half to twenty-three and one-half weeks of feeding. None of the animals in this group showed any evidence of gross atheromatous lesions of the aorta.

*Low Fat Diet Plus Stilboestrol Implants* Three chickens died early in the course of the experiment, at one, two and one-half, and six weeks of feeding. Three were sacrificed after nineteen weeks of feeding and three after twenty-three weeks of feeding. Five chickens showed atheromatous lesions of the aorta. Of the three chickens which were maintained on the low fat diet for one month prior to implantation of stilboestrol, two showed lesions of the aorta. Of the six in which the implantation was done at the same time that the animals were first placed on the low fat diet, three showed lesions. Two of the six died too early following the implantation to be considered as having had any lipemia as a result of the implantation. Considering the group as a whole, three

TABLE IV ARTERIOSCLEROSIS IN CHICKENS FED LOW FAT CHICK STARTER MASH SUPPLEMENTED WITH VITAMINS A, B, D AND E, 25 MG PELLETS OF STILBOESTROL WERE IMPLANTED AT THREE TO FOUR WEEK INTERVALS

WEEKS FED	AGE AT TIME OF DEATH (WK)	DEGREE OF ATHEROMA OF AORTA		LIVER, PRESENCE OF EXCESS FAT
		THORACIC	ABDOMINAL	
23	29	0	½	0
23½	29½	0	0	F
23½	29½	0	0	0
23½	29½	½	½	0
1	7	0	0	0
2½	8½	0	0	0
6	13	½	0	F
19	25	½	½	F
19	25	½	½	0
19	25	0	0	S F

F Fatty liver

S F Slightly fatty liver

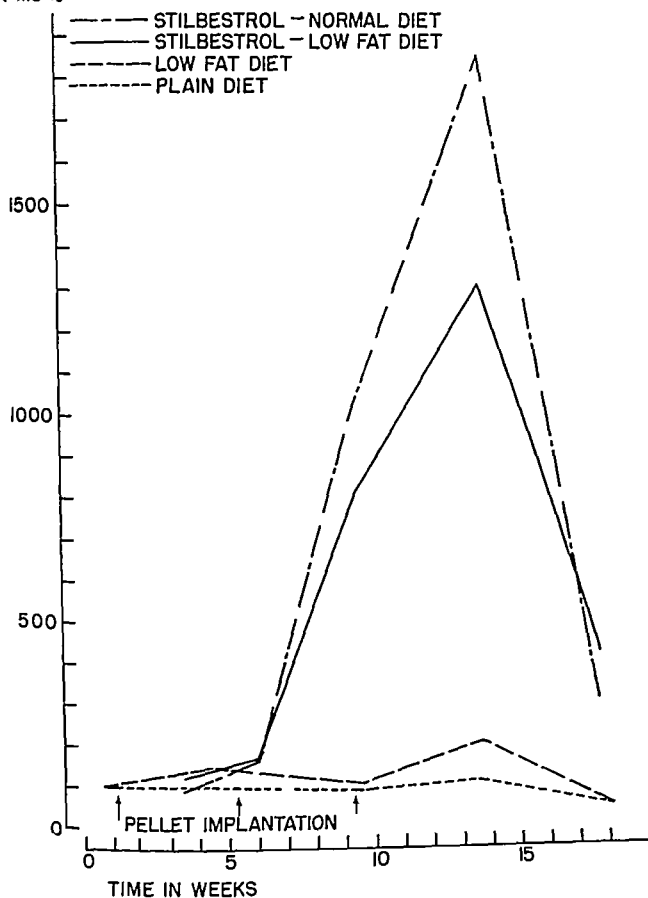
TOTAL  
CHOLESTEROL  
IN MG %

Fig. 1

birds had lesions in both the thoracic and abdominal aorta and of the other two one had lesions in the thoracic portion and one in the abdominal portion of the aorta. The lesions were slight grading from  $\frac{1}{4}$  to 1 and consisted of flat nonraised yellow areas and whitish areas

*Summary* The low fat control birds showed no gross atherosclerotic lesions of the heart or aortas, while atheromatous lesions were seen in five of the chickens in the low fat, stilboestrol implant group. There were lesions in 80 per cent of the chicks receiving ordinary mash with stilboestrol implants, while 40 per cent of the control birds on ordinary mash and water showed lesions. The lesions in the control group were almost entirely in the abdominal portion of the aorta, whereas those seen in the stilboestrol implanted groups were present in both the thoracic and abdominal aorta and were most prominent in the thoracic portion of the aorta.

*Fatty Livers*—Fatty livers were observed in two of the animals on the normal diet with stilboestrol implants and in three in the low fat, stilboestrol group. None of the normal controls had a fatty liver, but one of the low fat controls had a very slightly fatty liver.

*Body Weight*—It was observed that the birds on the normal mash diet with stilboestrol implants were considerably heavier than the normal controls and on autopsy there was a great deal of fat in the tissue depots. The birds on the low fat diet with stilboestrol implants were somewhat lighter than the low fat controls, and both groups were lighter than the normal controls. All birds in the low fat group had very little depot fat.

*Blood Cholesterol Levels*—In Fig. 1 we have plotted average blood cholesterol levels for the four groups of birds in this experiment. The value for the low fat stilboestrol group is made up of the values from the three birds which were on the low fat diet for one month prior to implantation. It will be seen that these values are higher than those obtained when the implantation was done at the same time that the animals were placed on the low fat diet (Table V).

TABLE V COURSE OF AVERAGE TOTAL SERUM CHOLESTEROL IN MILLIGRAMS PER CENT IN VARIOUS GROUPS

GROUP	DATE							
	2/10	3/17	4/7	4/14	4/25	5/19	6/16	7/14
Normal diet controls	88	114		119		94	109	
Low fat diet controls	94	112		149		111	205	
Normal diet stilboestrol implants			90		168	1036	1845	298
Low fat diet stilboestrol implants*			113		173	813	1293	411
	DATE							
	5/22	9/22	10/20	11/14	12/15			
Low fat diet stilboestrol implants†	88	167	647	729	544			

\*Pellets implanted after four weeks on low fat diet pellet implantations 3/2, 4/23, 5/23

†Pellets implanted at commencement of low fat diet pellet implantation 5/2, 9/23, 10/23

A possible explanation of this discrepancy is found in the work of Riddle and Senum<sup>10</sup> who found marked fluctuations from day to day in the blood lipids of birds treated with estrogenic substances.

The blood cholesterol levels for the normal control group and for the low fat control group were relatively stable during the experimental period. The latter group tended to show blood cholesterol levels somewhat higher than those for the normal control group. In the stilboestrol implanted birds, the first elevation of blood cholesterol was observed approximately four weeks after the first

TABLE VI CHANGES IN BODY WEIGHTS IN POUNDS IN VARIOUS GROUPS

GROUP	DATE				
	2/10	4/0	5/4	6/4	7/16
Normal diet controls	1.50	4.16	4.5	3.97	4.44
Low fat diet controls	1.50	2.80	3.5	3.5	3.44
Normal diet stilboestrol implants	1.50	4.1	4.10	3.87	4.18
Low fat diet stilboestrol implants	1.50	2.90	1.0	3.0	3.20

	DATE			
	8/5	9/2	10/2	1/5
Low fat diet stilboestrol implant f	1.50	2.00	3.00	3.0
Low fat diet controls	1.50	2.0	4.0	3.50
Normal diet controls	1.50	2.0	4.0	4.00

Pellet implanted 1 after four weeks on low fat diet

f Pellet implanted at start of low fat diet

implantation of stilboestrol pellets and reached its peak four weeks after the third and last implant. A peak value of 1800 mg. per cent of cholesterol was observed for the normal diet stilboestrol implant birds and of 1300 mg. per cent for the low fat, stilboestrol implant birds. The blood cholesterol fell sharply from the peak values reached and approached normal levels within four weeks.

In summary, the animals maintained on a low fat diet with vitamin supplements showed a tendency to slightly higher blood cholesterol levels than did the control birds. The implantation of stilboestrol pellets resulted in a massive lipemia in birds on a normal and on a low fat diet. The resulting lipemia was greater in the birds on the normal diet. Depleting body fat by maintaining the chickens for one month on a low fat diet prior to implantation of stilboestrol did not interfere with the chickens' ability to show a marked lipemia following the exhibition of the stilboestrol.

#### DISCUSSION

Our results are in accord with those of other observers who have found that estrogenic substances will result in a massive lipemia and hypercholesterolemia in the egg-laying vertebrates including, in this case, the chicken<sup>8, 11</sup>. We have been able also to confirm unequivocally the report of Chaikoff and co-workers<sup>14</sup> concerning the atherosclerotic action of stilboestrol in the chicken. Although the chickens which we used were younger than those employed by Chaikoff's group, nevertheless 40 per cent of our normal control birds showed the so-called spontaneous atherosclerosis in the aortas. The fact that the stilboestrol-treated birds showed lesions predominantly in the thoracic aorta and that their lesions were more severe than those in the control group makes it clear that the lesions in these birds were due to the action of stilboestrol and were not of the spontaneous type.

We also observed that maintaining birds for a long period of time on a low fat diet did not result in any appreciable lowering of the blood cholesterol below the normal control level for this species. In fact the blood cholesterol of these birds rose to levels slightly above the normal. However it is striking that despite this these birds seemed to be conspicuously free of the spontaneous atherosclerosis seen in control birds fed ordinary mash.

The implantation of stilboestrol pellets in birds on a low fat diet either at the commencement of this diet or after four weeks on the diet resulted in a

massive lipemia only slightly lower than that elicited by stilboestrol in birds on a normal diet. Further, a high proportion of the stilboestrol implanted birds on the low fat diet showed atherosclerotic lesions of an induced nature, whereas the low fat control birds were conspicuously free of atheroma. These findings indicate that it is impossible in the chicken to lower the normal level of blood cholesterol by rigidly excluding cholesterol and fat from the diet. This is in agreement with the work of other observers on the chicken and on man.<sup>21</sup> Further, the results indicate that under the stimulus of a substance which tended to produce a lipemia, chickens on a low fat diet responded almost as well as those on a normal diet.<sup>6</sup> Normal chickens which received stilboestrol showed remarkable lipid accumulations in the fat depots. Conversely, both the low fat controls and the low fat, stilboestrol implant birds showed a marked scarcity of body fat. These results indicate that under the stimulus of stilboestrol the chicken can mobilize and store great quantities of fat from ingested fats when available, and probably from carbohydrates and proteins in the diet. When the diet is low in fat, the sources of the lipemia are probably the body stores of lipids, and then the intermediate substances in carbohydrate and protein metabolism. This process is not seriously interfered with by preliminary depletion of fat by means of a low fat diet. Bloch and co-workers<sup>22, 23</sup> have demonstrated that acetic acid can serve as a precursor for cholesterol and that the site of conversion is most likely in the liver. Thus both carbohydrate and protein can serve as precursors for cholesterol through intermediary substances such as pyruvic acid.

Fleischmann and Friedl<sup>1, 18</sup> have presented evidence to the effect that the estrogen-induced lipemia of chickens can be completely prevented by the simultaneous administration of thyroid substances. They also showed that the total body cholesterol of estrogen-treated chickens is not greater than that of controls. Their experiments were of short duration and hence they were unable to observe the very marked accumulation of depot and body fat which we observed in our stilboestrol implanted chickens on a normal diet. While we did not determine total body cholesterol on these animals, it is highly probable that the cholesterol content of the body, in common with the lipids, was elevated.

Stilboestrol, in the egg laying vertebrates such as the pigeon, duck, sparrow, and chicken and in the egg laying fish and frogs, results in an elevation of the blood cholesterol. It is also generally agreed that the plasma cholesterol of mammals and of women increases during pregnancy. In the pregnant women the increase amounts to 50 to 100 per cent over the nonpregnant concentration.<sup>24</sup> Stilboestrol does not have a similar action in the rat.<sup>20</sup> Androgenic substances do not appear to have any clear cut effect on the levels of the blood cholesterol.<sup>1</sup>

We wish to suggest the possibility that the action of the thyroid substances<sup>19</sup> and estrogens is probably through the liver, by raising or lowering the thermostat setting for the blood level of cholesterol.<sup>32</sup> The liver will maintain this level of blood cholesterol even in the face of a very low intake of dietary cholesterol and fat by synthesizing cholesterol from the other dietary substances. Thus it appears futile to expect that limitation of cholesterol and fat in the diet will lower the normal blood cholesterol level. Furthermore, if abnormal influences are operating to raise the blood level for cholesterol, restriction of cholesterol in



the diet will only partially counter this effect, because of the ability of the body to mobilize and synthesize cholesterol from other sources. Thus we observed a massive lipemia and cholesterolemia and the development of atherosclerosis in animals under the influence of stilboestrol and on a diet very low in fat.

Stilboestrol probably acts to produce atherosclerosis through its cholesterolemic effect. We have previously reported<sup>21</sup> that in chickens fed cholesterol the occurrence of experimental atherosclerosis is related to the occurrence of a hypercholesterolemia and is roughly proportional in severity to the degree of hypercholesterolemia present. In this experiment the lipemia observed in low fat, stilboestrol implant birds was less than that observed in the control diet stilboestrol implant birds, and the degree of atherosclerosis observed was correspondingly less.

It is of great interest, however, that chickens maintained on a low fat diet and used as controls showed no gross atheroma whatsoever during the experimental period, whereas a group of controls on normal mash showed a 40 per cent incidence of atheromatous lesions. This finding occurred in spite of the fact that the blood cholesterol levels of the low fat birds were slightly higher than those of the normal controls. We do not wish to present this preliminary finding as more than suggestive. Our final conclusion on this point must await termination of the long term, as yet uncompleted experiment now in progress.

#### SUMMARY

The implantation of stilboestrol pellets resulted in a marked hyperlipemia and hypercholesterolemia in chickens on a normal diet and on a specially prepared low fat diet.

The cholesterolemia observed in the stilboestrol implanted chickens on the normal diet was somewhat higher than that observed in the animals on the low fat diet.

The control animals on the low fat diet showed levels of blood cholesterol slightly higher than those of the normal controls.

Atherosclerosis of the induced type was observed in a high proportion of the stilboestrol treated chickens in both the normal diet and low fat diet groups.

Spontaneous atherosclerosis occurred in 40 per cent of the normal control animals and in none of the controls on the low fat diet.

A concept of the mechanism of stilboestrol action and of the control of the normal blood cholesterol is presented.

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## EFFECTS OF TETRAETHYLAMMONIUM CHLORIDE ON BLOOD FLOW IN THE EXTREMITIES OF MAN

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ALTHOUGH the vasodepressor effects of the tetraethylammonium ion had been observed years ago by Marshall<sup>1</sup> and by Trendelenburg<sup>2</sup> the site of action was not known until recently. Acheson and Moe<sup>3</sup> and Acheson and Pereira<sup>4</sup> in 1946 showed that the drug caused its effect not by action on the heart, vascular smooth muscle, or medullary vasomotor centers but by blocking the ganglia in the efferent pathways of the sympathetic vasoconstrictor nerves. Acheson and Moe<sup>3</sup> measured the blood flow in the femoral artery of the dog by means of a flow cannula and a differential manometer. They found that while the intra-arterial injection of tetraethylammonium chloride caused no increase in blood flow, the intravenous injection caused a marked increase in blood flow. Berry and associates<sup>5</sup> showed that tetraethylammonium chloride caused a rise of skin temperature in man equal to or greater than that caused by sympathetic block. By the use of the plethysmograph Collier and associates<sup>6</sup> demonstrated an increase in peripheral blood flow following intravenous administration of tetraethylammonium chloride to patients suffering from various vascular and allied disorders. In one subject the blood flow in the foot increased from 0.24 to 5.2 c.c. per minute per 100 Gm. of tissue following the intravenous administration of 500 mg. of tetraethylammonium chloride.

The purpose of this study was to determine the effect of tetraethylammonium chloride on the blood flow in the upper and lower extremities of healthy human beings in a relatively warm environment (temperature ranging between 80 and 85°F) and to establish a basis for comparison with subsequent studies on various abnormalities of the vascular system in patients.

Seven healthy young adults, four men and three women whose ages ranged from 22 to 30 years and whose weights ranged from 118 to 190 pounds (53.5 to 86.2 kilograms), were studied. The room temperature varied from day to day between 80 and 85°F but it did not vary more than 1°F, during any single period of observation. The plethysmographs used in this study were those designed by Berry and associates. The plethysmographs were connected to compensating spirometer recorders and blood flow curves were recorded optically. The arm plethysmograph included the hand and forearm to 1 inch (2.5 cm.) above the olecranon process, and the leg plethysmograph included the foot and leg to 1 inch below the tibial tuberosity.

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The procedure employed in this study was as follows. The subjects were instructed not to eat or smoke for at least ninety minutes before the test. All four extremities were comfortably sealed in the plethysmographs and sufficient time was allowed for adaptation. Control determinations of blood flow were made for twenty to thirty minutes thereafter. Tetraethylammonium chloride was then administered intravenously at the rate of 100 mg per minute. Six individuals were given 300 mg each and one subject, a man weighing 190 pounds, was given 450 mg of tetraethylammonium chloride. The drug was given slowly in order to avoid unpleasant reactions such as precipitous fall in blood pressure, anxiety, and so forth. Blood flow was determined again immediately after completion of the injection and at regular intervals for thirty-five to forty-five minutes thereafter.

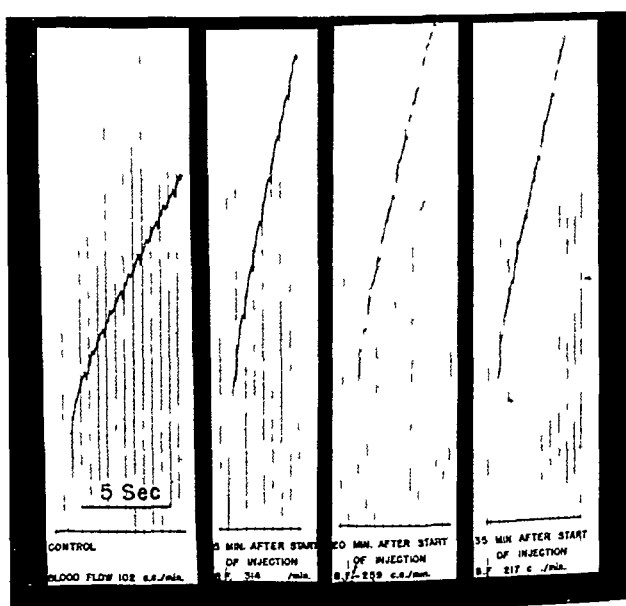


Fig 1—Blood flow in the leg before and after intravenous administration of tetraethylammonium chloride

A typical series of blood flow curves from the forearm which are representative of the changes observed in all the extremities is shown in Fig 1. The first curve is a record of control flow, the total flow was 102 cc per minute. The next flow curve was taken five minutes after the injection was started. It shows a blood flow of 314 cc per minute, a significant increase over the control flow. The next two curves were taken at twenty and thirty-five minutes, respectively, after the injection was started, they show a gradual reduction of the augmented flow, but it is still higher than the control value.

The typical response to tetraethylammonium chloride is shown in Fig 2. After the control blood flow was established, 300 mg of the drug were given intravenously. The blood flow increased immediately and reached a maximum about five to six minutes after the injection was started. The flow at the peak

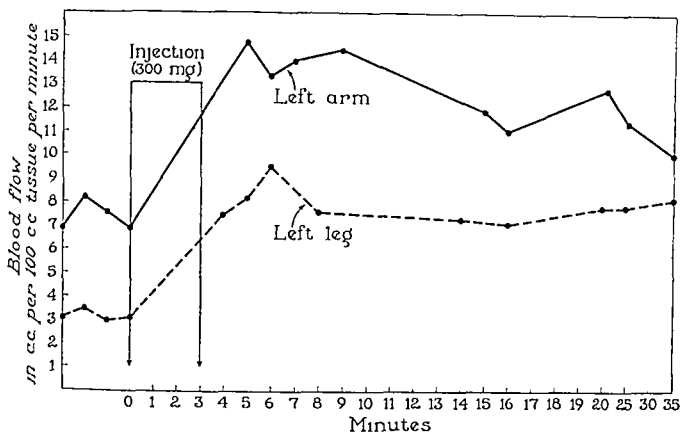


Fig 4.—Changes in blood flow in the upper and lower extremities as a result of intravenous injection of tetraethylammonium chloride.

of the increase in the leg was 197 per cent higher than the control value. The flow then gradually decreased but even at the end of the observation (thirty five minutes after injection) the flow was still greater than the control.

TABLE I EFFECTS OF INTRAVENOUS ADMINISTRATION OF TETRAETHYLAMMONIUM CHLORIDE ON THE BLOOD FLOW IN THE EXTREMITIES

SUBJECT	FLOW (CC PER 100 CC OF TISSUE PER MIN)				INCREASE IN FLOW (%)		DOSE OF TETRAETHYL AMMONIUM CHLORIDE (MG)
	CONTROL		MAXIMUM AFTER TETRAETHYLAMMONIUM CHLORIDE				
	FOREARM	LEG	FOREARM	LEG	FOREARM	LEG	
1	73	32	147	95	101	197	300
2	17	17	34	51	100	200	300
3		45	--	72	--	60	300
4	47	18	100	40	113	122	300
5	67	38	105	75	57	97	450
6	50	--	110	--	120	--	300
7	40		83	--	108	--	300
Average	49	30	97	67	100	135	

The results obtained from study of the seven persons are summarized in Table I. The average blood flow in the forearms of all the subjects before the administration of tetraethylammonium chloride was 49 cc per 100 cc of limb volume per minute, and it ranged from 17 to 73 cubic centimeters. After the injection of tetraethylammonium chloride the blood flow increased in every case. The maximal increase occurred between five and fifteen minutes after injection (average, about nine minutes). In all the subjects the maximal blood flow in the forearms after the injection of tetraethylammonium chloride averaged 97 cc per 100 cc of limb volume per minute and ranged from 34 to 147 cubic centimeters. This represents an average increase of 100 per cent over the control flow,

with a range of increase of 57 to 120 per cent. The control blood flow in the legs, before the administration of tetraethylammonium chloride averaged 30 cc per 100 cc of limb volume per minute and ranged from 17 to 45 cubic centimeters. After injection of the drug, the blood flow in the legs also increased in every case and reached a maximum at the same time as in the arms. The average maximal blood flow in the legs, after injection of tetraethylammonium chloride, was 66 cc per 100 cc of leg volume per minute, the range was from 40 to 95 cubic centimeters. This represents an average increase of 135 per cent over the control value with a range of from 60 to 200 per cent. The increased blood flow in both upper and lower extremities gradually regressed, nevertheless, at the termination of the observation thirty-five to forty-five minutes after the administration of tetraethylammonium chloride, the flow was still higher than the control value. A rise in cutaneous temperature accompanied the increase in blood flow.

In addition to the changes in blood flow, the subjects experienced numbness and tingling, tachycardia, a metallic taste, dryness of the mouth, and variable disturbances of vision with impairment of accommodation.

#### SUMMARY

The effects of intravenously administered tetraethylammonium chloride on the blood flow in the upper and lower extremities of healthy subjects were studied plethysmographically by the use of the compensating spirometer recorder. In the presence of vasodilatation due to a relatively warm environment of 80 to 85° F, tetraethylammonium chloride produced a substantial increase in blood flow in the upper and lower extremities. The average increase in blood flow was 100 per cent in the forearms and 135 per cent in the legs. In addition to the increase in blood flow, disturbances of vision with impairment of accommodation, metallic taste and dryness of the mouth, and increase in heart rate occurred after injection of tetraethylammonium chloride.

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# FALSE POSITIVE TESTS FOR SYPHILIS—A FURTHER STUDY OF THEIR INCIDENCE IN SPOROZOITE INDUCED VIVAX MALARIA

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THE program of testing malarious prisoners in the United States which was initiated in 1944 by the National Institute of Health has afforded exceptional opportunities to study the causative relationship of malaria to false positive tests for syphilis. The use of experimental sporozoite induced infections has made it possible to demonstrate the absence of nonspecific serum reactions in large groups of selected nonsyphilitic individuals before exposure to malaria and to examine successive serum specimens through all stages of the disease. Serums have been sent to the Army Medical Department Research and Graduate School where they have been subjected to standard and experimental tests for syphilis. The first serologic studies which were confined to flocculation tests for syphilis, provided material for an earlier report<sup>1</sup>. It was found that the standard flocculation tests in general yielded a high incidence of nonspecific reactions following infection with *Plasmodium vivax* whereas a microfloculation test with cardiolipin antigen<sup>2</sup> showed relatively few such reactions.

In continuing these serologic studies it has been our purpose to extend the investigation to complement fixation as well as flocculation tests for syphilis and to include certain more recently developed tests that employ cardiolipin antigens.

## MATERIALS AND METHODS

Subjects for this study were selected from white male volunteers; only those individuals were considered whose preliminary examinations showed no clinical anamnestic or serologic evidence of syphilis. Successive specimens of serum were obtained from each volunteer before his inoculation with malaria by mosquito bite at two to three day intervals during and immediately following attacks and at weekly intervals during periods of latency. The total time of study in many of the volunteers extended over a period of eighteen months. The serums were preserved in tubes containing dried Merthiolate sufficient to give a final concentration of 1 mg. per milliliter of serum. They were shipped by ordinary mail to the Army Medical Department Research and Graduate School where they were subjected to eight serologic tests for syphilis. Four of these tests employed ordinary tissue extract antigens the remainder were carried out with cardiolipin antigens. The former group included the Kahn standard test, the Kline exclusion and Mazzini microfloculation test and the standard Kolmer complement fixation test. The latter group consisted of the Reim-Bussack and VDRL microfloculation tests, a Kolmer complement fixation test<sup>3</sup> and a quantitatively standardized complement fixation procedure<sup>4</sup> which has been designated the F-1-30 test since the end point of 30 per cent hemolysis is employed in its standardization. A total of 6403 serums were examined in this manner. 514 serologic tests for syphilis were performed.

\*The serologic tests which form the basis for this study were performed by Robert W. Santora and Harriet M. Boylston whom grateful acknowledgment is made.

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## RESULTS

The present report is based upon results obtained with 104 individuals who experienced one or more attacks during the course of their malarial infections. Of this group, 78 had been infected with sporozoites of the Chesson strain of *P. vivax*, 17 with sporozoites of the St. Elizabeth strain, and 9 with sporozoites of both strains simultaneously. Collectively these subjects experienced 307 attacks of malaria.

The incidence of false positive tests for syphilis was determined from the number of subjects who developed persistent nonspecific reactions after infection with malaria. To be considered as a false positive reactor, it was required that an individual show reactions of plus-minus or greater in at least two successive serums. Of the 104 infected subjects, 75 (72 per cent) developed such false positive reactivity in one or more tests at some time during the course of the disease. This composite group represented 51 (65 per cent) of those infected with the Chesson strain, 15 (88 per cent) of those infected with the St. Elizabeth strain, and 9 (100 per cent) of those infected with both strains. The number of individuals included in the last two groups was too small to permit accurate conclusions regarding the relative incidence of false positive reactors. The

TABLE I. RESULTS OBTAINED IN EIGHT SEROLOGIC TESTS FOR SYPHILIS USING SUCCESSIVE SERUMS DRAWN BEFORE, DURING, AND AFTER A PRIMARY ATTACK OF SPOROZOITE-INDUCED CHESSEON STRAIN VIVAX MALARIA.

DAY TESTED, RELATIVE TO EXPOSURE DATE (DAY 0)	KAHN STAND ARD	KLINE EXCLU SION	MAZZINI FLOCCU LATION	KOLMER STAND ARD	REIN BOSSAK	VDRL	KOLMER CARDIO LIPIN	EP 50 CARDIO- LIPIN
- 11	---	-	-	-	-	-	-	-
+ 9	---	-	-	-	-	-	-	-
+ 11	Parasitemia patent	-	-	-	-	-	-	-
+ 15		-	-	-	-	-	-	-
+ 22		4 4 4	±	3	-	-	-	-
+ 26		4 4 4	1	1	-	-	-	-
+ 29	1 2 2	4	-	±	-	-	-	-
+ 37	- ± 1	4	-	±	-	-	-	-
+ 41	---	1	-	-	-	-	-	-
+ 47	---	-	-	-	-	-	-	-

frequency with which such reactors were encountered in infections with the St. Elizabeth strain appeared high in comparison with the more extensive previous experience<sup>1</sup> in which 61 per cent of 80 similarly infected subjects developed nonspecific reactivity. If this earlier observation is taken as the more trustworthy, false positive reactors would appear to occur with approximately equal frequency in infections with the Chesson or St. Elizabeth strains. The exceptionally high percentage of reactors among the few individuals who were infected with both strains may represent a significant difference, but the findings require confirmation in a more extensive series.

Table I illustrates the results obtained in the eight tests for syphilis using successive serums drawn from an individual before, during, and after a primary attack due to the Chesson strain. In this case parasitemia became patent on the



TABLE II. SUBJECTS DEVELOPING FALSE POSITIVE REACTIVITY IN SEROLOGIC TESTS FOR SYPHILIS DURING SPOKOZOFF INDUCED VIVA MALAIA\*

STAIN OF P. VIVAX	ATTACK	NUMBER OF SUBJECTS TESTED	RAHN STANDARD		KLING EXCLUSION		MAZZINI FLOCCULA TION		KOLMER STANDARD		REIN ROSSA		VDRL		KOLMER CARDIO LIPIN		E. P. 50 CARDIO LIPIN	
			NO	%	NO	%	NO	%	NO	%	NO	%	NO	%	NO	%	NO	%
Chesson	1	78	48	62	23	30	15	19	12	15	9	12	11	14				
	2	59	19	32	6	10	2	3	2	3	2	3						
	3	43	10	23	7	16	1	2			1	2	1	2				
	4	28	5	18	3	11	1				1							
	5	15	2	13	1	7												
	6	10																
	7	6	1		1													
	8	5	1															
	9	4																
	10	4																
	11	3	1						1									
	12	2																
St Elizabeth	1	17	15	88	11	65	7	41	2	1					1	6	1	6
	2	8																
	3	1																
Chesson and St Elizabeth	1	9	8	89	9	100	4	44	2	22			2	22				
	2	7	5	71	1	14												
	3	6	1	17														
	4	2																

\*Blank spaces represent absence of reacting individuals

eleventh day after inoculation. On the twenty-second day, nonspecific reactions appeared in all four of the tests that employed ordinary tissue extract antigens. They persisted for intervals varying from five days for the Mazzini test to twenty days for the Kline exclusion test. In marked contrast were the results obtained in the tests employing cardiolipin antigens. The Rein-Bossak and VDRL microflocculation tests as well as the Kolmer and E P 50 complement fixation tests remained negative throughout.

False positive reactions were not ordinarily encountered in advance of the fever or parasitemia of an attack. Occasional exceptions to this rule were observed when relapses occurred before the seropositivity due to preceding attacks had subsided. Reactions appeared, on the average, 8.7 days following the onset of patent parasitemia, the interval varying within the limits of 0 to 39 days depending upon the individual and upon the specificity of the test. These values correspond closely to the mean interval (8.3 days) and range (0 to 30 days) observed in the previous study<sup>1</sup> in which the majority of infections were due to the St. Elizabeth rather than to the Chesson strain. It should be pointed out that while nonspecific reactivity in most individuals was transitory and of low degree, reactions as high as 4 plus were encountered with all the tests. Reactivity due to single attacks of malaria persisted for periods varying from 2 to 77 days, the observed duration again reflecting differences in the individual and in the specificity of the test. False positive reactions of longer duration (23 to 181 days) were observed in twenty-one instances when one or more successive relapses extended the seropositivity due to an earlier stimulating attack.

A comparative analysis of the tests with regard to their specificity for syphilis was based upon the relative number of subjects who became false positive reactors as defined in the foregoing. Separate analyses were made for the three groups that were infected, respectively, with the Chesson, the St. Elizabeth, and the combined strains. These are summarized in Table II which gives the numbers of false positive reactors encountered with each test in successive malarial attacks. As indicated in the previous study,<sup>1</sup> their incidence was highest in primary attacks, the numbers decreasing progressively with succeeding relapses. Among the procedures employing ordinary tissue extract antigens, the Kahn standard test yielded the highest number of nonspecific reactors and the Kolmer complement fixation test the lowest. Of the tests with cardiolipin antigens, the Rein-Bossak and VDRL microflocculation tests showed still fewer reactors and the Kolmer and E P 50 complement fixation tests all but eliminated such reactors. It is noteworthy that the same individual accounted for the only reactions which were observed in the cardiolipin complement fixation tests. These reactions attained the maximal degree of 4 plus during a six day interval. They were paralleled by equally strong reactions in all the tests with ordinary antigens, but no reactivity whatsoever was encountered in the cardiolipin microflocculation tests. All the serologic findings in this case were corroborated by repetition.

Some additional evidence of the relative specificity of the tests could be obtained by comparing them with regard to (a) the interval between onset of pat

TABLE III. INTERVAL FROM ONSET OF PATENT PARASITEMIA TO APPEARANCE OF FALSE POSITIVE REACTION AND DURATION OF REACTION IN FIGHT SEROLOGIC TESTS FOR SYPHILIS

SEROLOGIC TEST FOR SYPHILIS		KAHN STAND- ARD	KLINE EXCLU- SION	MAZZINI FLOCCU- LATION	KOLMER STAND- ARD	LEIN- BOSSAK	VDRI	KOL- MER CAR- DIO- LIPIN	E.P. 50 CAR- DIO- LIPIN
Interval parasitemia to false positive reaction (days)	Mean	7.4	5.3	9.0	10.1	1.5	1.1		
	Range	(0-15)	(0-35)	(-20)	(0-39)	(0-22)	(0-26)	(6)	(6)
Duration false positive reaction (days)	Mean	11.4	13.3	8.4	13.6	10.8	5.8		
	Range	(-77)	(2-28)	(1-20)	(3-41)	(3-23)	(3-13)	(6)	(6)

ent parasitemia and the development of the respective false positive reactions and (b) the duration of these reactions. The mean value calculated for each test is given with the range in Table III. It is of interest that the tests in which nonspecific reactions appeared earliest and lasted longest were those which showed the highest incidence of false positive reactions (compare Table II). The occurrence of only one reactor in the complement fixation tests with cardiolipin antigen prevented comparing them with the other tests on this basis but left no doubt as to their superior specificity.

The present findings support the conclusion that in vivax malaria complement fixation tests for syphilis show a higher degree of specificity for syphilis than do the flocculation tests. This superiority was evident regardless of whether results with ordinary tissue extract or with cardiolipin antigens were compared. The introduction of cardiolipin antigens reduced the incidence of false positive reactions in microfloculation tests and virtually eliminated such reactions in complement fixation tests. The specificity of the cardiolipin tests was striking in view of their high sensitivity in syphilis. The observations have obvious implications regarding the serodiagnosis of syphilis particularly where the possibility of a current or recently subsided malarial infection arises.

#### SUMMARY AND CONCLUSIONS

In continuing studies of malaria as a cause of false positive tests for syphilis 6403 specimens of serum were collected at suitable intervals from 104 nonsyphilitic individuals with sporozoite induced vivax malaria. The serums were subjected to eight serologic tests for syphilis which included flocculation and complement fixation procedures employing either ordinary tissue extract or cardiolipin antigens, 51,224 tests were performed in all.

Seventy-five (72 per cent) of the 104 subjects developed false positive reactivity in one or more of the tests for syphilis at some time during the course of their malarial infections. Nonspecific reactions appeared from 0 to 39 days following the onset of patent parasitemia and lasted from 2 to 181 days. Their time of appearance and duration varied with the individual and with the specificity of the test.

Complement fixation tests for syphilis exhibited a higher degree of specificity for that disease than flocculation tests. Of the procedures employing ordinary tissue extract antigens, the Kahn standard test showed the highest incidence of false positive reactions and the Kolmer test the lowest. Cardiolipin antigens reduced the incidence of nonspecific reactions in microfloculation tests and virtually eliminated such reactions in complement fixation tests.

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# TRANSIENT POSITIVE WASSERMANN TEST FOR SYPHILIS IN ACUTE HEMOLYTIC ANEMIA

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COMPREHENSIVE studies of the incidence and nature of the biologic false positive reaction for syphilis with an exhaustive review of literature have been published recently by Davis, Mohr and co workers Beerman Rein and Elsberg, and Kolmer<sup>1</sup> The reader is referred to these excellent publications for more details

The case to be reported here is one of acute acquired hemolytic jaundice with spontaneous recovery following transfusions Transient positive Wassermann tests for syphilis were found during the acute hemolytic attack and became negative after the attack subsided

Since we have not found any record in the available literature of biologic false serologic reaction in the course of hemolytic anemia this case seems worthy of reporting

## CASE REPORT

Patient J Z, a 50 year old white man entered the hospital on Sept 23, 1946 with complaints of yellow discoloration of the skin The family and the patient's own past history revealed no history of jaundice at any time nor of any exposure to chemicals The illness had begun a month previously at which time the patient was told by a physician that he had jaundice and an enlarged liver

Blood examination on Sept 19, 1946 showed hemoglobin, 25 per cent red blood cells, 1,500,000 white blood cells, 9,400 differential count normal icteric index 24 At that time a slightly enlarged spleen was felt and a moderately enlarged axillary node was noted

At the time of the patient's admission to the hospital the skin and sclerae were strikingly yellow There was an enlarged firm movable slightly tender lymph node in the right axilla, about 5 cm. in diameter The spleen was felt 1 fingerbreadth below the left costal margin and the liver was felt 2 fingerbreadths below the right costal margin There was severe anemia (hemoglobin 3.5 Gm, red blood count 1,150,000) with a most pronounced reticulocytosis of 88 per cent and normoblastosis as well as macrocytosis Serum bilirubin was 4.3 mg per 100 ml and the van den Bergh reaction was delayed The red cell fragility test in saline solutions showed beginning hemolysis at 0.70 per cent NaCl and complete hemolysis at 0.50 per cent NaCl Sternal bone marrow aspiration revealed a markedly hypercellular marrow with marked increase of erythronormoblastic elements in the differential count (about 60 per cent of the total nucleated cell count of 500,000 in 1 cmm) The urinalysis showed traces of albumin and was negative for bile and sugar

On the basis of these findings the diagnosis of hemolytic anemia was made

In order to determine the possible etiologic factor of this hemolytic process additional studies were made The Wassermann reaction was reported + plus on Sept 24 1946, with the Kahn reaction negative The Donath Landsteiner test was performed on Oct 2, 1946 and was negative for the presence of specific cold hemolysins The lack of history of exposure to cold was also against the Donath Landsteiner mechanism of hemolysis Cold agglutinins were absent as well Agglutination tests for typhoid, paratyphoid A and B, brucellosis, and Proteus OX<sub>19</sub> were negative Also, the presence of malarial parasites

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TABLE I PERIPHERAL BLOOD STUDIES

DATE (1946)	HEMOGLOBIN (Gm PEP 100 cc)	DIFFERENTIAL COUNT OF WBC										PLATELETS (IN 1 CMM)	EFFICIENCY FES	BIOD SPEM	++++ Macrocystis, polycystis, tosis, polyehomast, basophilic stippling of red cells			
		RED BLOOD CELL COUNT (PEP 1 CMM)			WHITE BLOOD CELL COUNT (PEP 1 CMM)			NEUTROPHILS								MONOCYTES		
		SEGMENTED (PEP 1 CMM)	NON SEGMENTED	SEGMENTED	LYMPHOCYTES	EOSINOPHILS	BASOPHILS											
9/24	35	1,150,000	5,160	Transfusion of 500 cc of whole blood				3	70	16	2	1	8	175,000	88	++++		
9/25	45	1,330,000	Transfusion of 500 cc of whole blood				7	64	25	Transfusion of 500 cc of whole blood				4	2	150,000	5	+++
9/28	55	1,600,000	6,850	Transfusion of 500 cc of whole blood				2	64	26	2	2	4	280,000	5	+++		
10/2	665	2,090,000	Transfusion of 500 cc of whole blood				2	64	26	2	2	4	280,000	5	+++			
10/8			Transfusion of 500 cc of whole blood				2	64	26	2	2	4	280,000	5	+++			
10/9	70	2,700,000	5,800	Transfusion of 500 cc of whole blood				2	64	26	2	2	4	280,000	5	+++		
10/11			Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			
10/12	95	1,000,000	Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			
10/16			Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			
10/17	11	3,900,000	Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			
10/22	115	4,170,000	Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			
10/23			Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			
10/25	135	1,430,000	Transfusion of 1,000 cc of washed red cells				2	64	26	2	2	4	280,000	5	+++			

could not be shown. Blood cultures were negative. Biopsy study of the axillary node revealed granulomatous lymphadenitis with multiple abscess formation; no specific etiology could be suggested, but lymphomatous diseases were ruled out.

Because no underlying disease could be shown or any known exposure incriminated, the hemolytic process was defined as acute acquired hemolytic anemia of unknown origin.

TABLE II. SPINAL BONE MARROW STUDIES

Date	9/24/46	10/23/46
Total nucleated cell count (per 1 c mm.)	500,000	150,000
Number of megakaryocytes (per 1 c mm.)	66	2
Differential count (%)		
Myeloblasts	0.8	0.5
Promyelocytes	2.0	1.5
Myelocytes neutrophils	18.0	25.5
Myelocytes eosinophils	0.4	2.5
Non segmented neutrophils	6.0	15.5
Segmented neutrophils	32	12.5
Segmented eosinophils	14	1.5
Segmented basophils	-	0.5
Lymphocytes	1.0	8.0
Hematogones	0.5	1.0
Plasma cells	0.5	0.5
Reticulum cells	0.4	-
Proerythroblasts	12	-
Erythroblasts	12	5.5
Normoblasts	53.0	25.0

The patient was given repeated transfusions with excellent hematologic and clinical response. There followed a progressive improvement of the patient's general condition; the hepatosplenomegaly gradually receded; the red blood count increased; the reticulocytosis subsided, and the fragility of red cells returned to normal range. On Oct. 12, 1946, the hemoglobin was 9.5 Gm and the red blood count 3,000,000. On Oct. 14, the Wassermann and Kahn were negative and remained so on all subsequent examinations up to the time of writing.

The patient was discharged in good condition on Oct. 25, 1946, with hemoglobin 13.5 Gm per 100 cc and red blood count 4,430,000 per 1 cubic millimeter. The bone marrow examination repeated on Oct. 25, 1946, showed essentially normal findings.

The patient returned to work and since then has been observed in the follow-up hematology clinic. The patient has remained in good health and no clinical or hematologic abnormalities have been noted.

#### COMMENT AND SUMMARY

A case of hemolytic anemia is reported in which a transient strongly positive Wassermann reaction was found with negative Kahn and Kline tests.

The Wassermann reaction declined gradually with the receding hemolytic process. When the patient was first examined at the height of the hemolytic process (hemoglobin, 3.5 Gm in 100 cc; reticulocytes, 88 per cent) the Wassermann reaction was 4 plus; ten days later, with 5.5 Gm hemoglobin and 58 per cent reticulocytes, the Wassermann reaction was 3 plus; finally, after the following ten days, with 10 Gm hemoglobin and 60 per cent reticulocytes, the Wassermann reaction was negative throughout.

Although we have not found any other report in the available literature of biologic false serologic reaction in the course of hemolytic anemia, the case recorded here is not the only one known to us. We have previously observed a

patient with chronic hemolytic anemia associated with Laennec's cirrhosis of liver and complicated by diabetes mellitus in whom positive serologic tests for syphilis gradually became negative after splenectomy. Similar unpublished observations were made by other authors<sup>2</sup> in cases of acute as well as chronic hemolytic anemia, in which both the Wassermann and the Kahn tests showed transient positive reactions.

It appears therefore that the occurrence of biologic false Wassermann reaction in hemolytic anemia might not be uncommon.

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# THE PURIFICATION OF DIPHTHERIA AND TETANUS ANTITOXIN BY THE USE OF PEPSIN

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## INTRODUCTION

INVESTIGATIONS by numerous workers have shown that antibodies are closely related to normal serum proteins and occur in the globulin fraction of serum. The chemical and immunologic behavior of antibodies and antitoxins has been reviewed by Kabat<sup>1</sup> in a paper dealing with the immunochemistry of proteins. It has been known for many years that certain antibodies are relatively resistant to destruction by proteolytic enzymes.<sup>2</sup> This property has been utilized by Parfentiev,<sup>3</sup> Pope<sup>4</sup> more recently by Gerlough<sup>5</sup> and by others to effect the purification of antitoxins.

Various proteolytic enzymes have been employed to purify antitoxins. Pick<sup>6</sup> followed at a later date by Mellanby<sup>7</sup> first studied the action of trypsin and pepsin upon diphtheria antitoxin. It is of interest to note in passing that in 1903 the first commercial patent was granted by the British Patent Office to Oliver Imray<sup>11</sup> for the purification of diphtheria antitoxin by the action of trypsin and also by the action of pepsin.

It was many years following the investigations of Pick and Mellanby that interest was again revived by Parfentiev in the use of enzymes to purify antitoxins. The studies of Parfentiev<sup>3</sup> were followed by the investigations of Pope<sup>4</sup> who reported that in addition to the use of pepsin satisfactory results could also be obtained with trypsin, papain, maltin (obtained from a commercial preparation of diastase), and fibrinolysin. Modern and Ruff<sup>12</sup> confirmed the observations of Pope concerning the peptic purification of diphtheria antitoxin. The enzymatic purification of tetanus antitoxin was confirmed by Sandoz and Richon<sup>13</sup> and also by Modern and Ruff.<sup>14</sup>

Coakill and co-workers<sup>1</sup> have developed a process to purify antitoxins using the enzyme Taka diastase. More recently Northrop<sup>16</sup> has prepared purified diphtheria antitoxin by means of digestion of the toxin-antitoxin complex with crystalline trypsin followed by fractional precipitation with ammonium sulfate.

This discussion would be incomplete without a brief statement concerning the effect of proteolytic enzymes upon antibacterial sera. Antibacterial antibodies behave somewhat differently toward peptic action than do antitoxic antibodies. Schultze<sup>17</sup> has observed that diphtheria, tetanus, and perfringens antitoxic sera are less extensively hydrolyzed than normal horse serum. On the other hand, antibacterial sera prepared against pneumococcus and streptococcus are hydrolyzed at the same rate as normal horse serum. Grabau<sup>18</sup> noted

<sup>1</sup>From the William H. H. Park Laboratory, Bureau of Laboratories, Department of Health.

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that type I antipneumococcus horse serum which was subjected to peptic action lost one-half of its antibody nitrogen with an accompanying loss of only one-fourth of the total nitrogen. The observations of Schultze and Giabar have been confirmed by van der Scheer and associates<sup>19</sup> in their electrophoretic examination of digested sera.

The pursuit of suitable methods for treating immune horse serum in order to avoid serum reactions in human beings has spurred the development of the enzymatic process for the purification of antitoxins. Weil, Parfentiev, and Bowman<sup>20</sup> were the first to study the antigenic qualities of horse serum antitoxin. They found that treating the antitoxic serum with pepsin impaired the antigenicity of the serum to a great extent.

The investigations of Parfentiev and associates were followed by the studies of Coghill and co-workers<sup>15</sup> on the use of the enzyme Taka diastase to eliminate horse serum specificity from antitoxins. The premise underlying the use of Taka-diastase is that the carbohydrate portion, which is associated with the pseudoglobulin fraction of horse serum, is responsible for serum reactions in human beings. Rimington<sup>21</sup> was the first to isolate and study the chemical properties of this polysaccharide. However, Coghill and Creighton, using the chemical procedures of Rimington, demonstrated the nonspecific nature of the carbohydrate associated with horse serum pseudoglobulin.

Kass, Scheerago, and Weaver<sup>22</sup> have investigated the effects of enzymic digestion upon the antigenic qualities of antitoxic and normal horse plasmas. Their plasmas were digested with *Aspergillus oryzae* diastase, malt diastase and pepsin. Using the Schultz-Dale technique in their anaphylactic studies on guinea pigs, they found that the digested antitoxins were antigenically related no matter which of the three enzymes mentioned was utilized for digestion of the horse plasmas.

There are available two general methods of purifying antitoxic sera by means of pepsin. These methods are outlined by Parfentiev and Pope, respectively. In the method of Parfentiev the unwanted proteins of antitoxic serum are digested at about pH 4.0\*. This is followed by the adsorption of nonantitoxic proteins present in the digest product†. In his process Parfentiev permits the digestion of an antitoxic serum to continue to a point where proteoses and other substances commonly associated with the enzymatic decomposition of proteins are produced. These substances as well as some pepsin which may remain must be adsorbed from the solution since they are not readily separated from the antitoxic proteins by the usual method, that is fractional precipitation with ammonium sulfate. In practice, various adsorbing agents are employed such as aluminum cream,<sup>24</sup> tricalcium phosphate,<sup>25</sup> or other similar substances. In a subsequent patent which Parfentiev obtained,‡ digestion of an antitoxic serum is carried out at pH 3.0 to pH 3.2 at 37° C. for several hours. This is again followed by adsorption with tricalcium phosphate in a manner similar to the procedure, previously described.

\*As outlined in U. S. Patent No. 2,065,196 (1936).

†As outlined in U. S. Patent No. 2,123,198 (1938).

‡Namely U. S. Patent No. 2,175,090 (1939).

In the procedure of Pope antitoxic serum is treated with pepsin for a short period of time at room temperature. This step is followed by rapid heating of the serum in the presence of a large quantity of a salt to some critical temperature at which temperature the unwanted proteins are coagulated. The antitoxic proteins remain in solution and may readily be separated from the coagulum by filtration or centrifugation. This is followed by the addition of more salt with the subsequent precipitation of the antitoxic proteins. This method is based on the following considerations. According to Pope diphtheria antitoxin is a complex molecular aggregate which consists of two parts. One part is readily denatured by heat, strong acids or alkalis and so on, while the other part which carries the antitoxic properties is not readily denatured. This theory has been substantiated for diphtheria antitoxic horse pseudoglobulin by Petermann and Pappenheimer<sup>6</sup> as well as for bovine pseudoglobulin.

It becomes evident at once from an examination of the two methods that basic differences exist between the procedure of Pulfantiev and that outlined by Pope. The former method depends upon the digestion of serum proteins with the concomitant production of substances usually associated with the enzymatic decomposition of proteins. The method of Pope consists in a disaggregation of the pseudoglobulin by partial enzyme action followed by heat, which results in the separation of antitoxic from nonantitoxic protein. In the latter instance, therefore, true digestion does not take place to any considerable extent.

#### PROCEDURES

The principal enzyme used for the preparation and purification of antitoxins on a commercial scale is pepsin. The use of pepsin has obviated the difficulty experienced in controlling the digestion process as well as the difficulty in separating the enzyme itself from the solution of antitoxin once its function in the purification process has been completed. By varying the pH value of the solution it is a simple matter to control digestion and to obtain a solution of antitoxin entirely free of peptic action. Moreover commercial preparations of pepsin of various digestive potencies and of reasonable purity may be purchased in sufficient quantities. For the foregoing reasons pepsin is employed in the following procedure to effect the purification of diphtheria and tetanus antitoxin.

*Processing of Plasma and Globulin*—The entire process given below was carried out in a jacketed vat having a capacity of 65 liters. Sample batches of antitoxic horse plasma or antitoxic pseudoglobulins were treated with pepsin.

- 1 Antitoxic plasma or antitoxic pseudoglobulin recovered from ammonium sulfate fractionation of immune horse plasma is diluted with a sufficient volume of physiologic salt solution to reduce the total solid content to 2 to 3 per cent.

- 2 Four tenths per cent phenol is added to the diluted plasma or globulin solution. The addition of phenol serves not only as a preservative but also aids in the heat denaturation process listed below.

- 3 The solution is then adjusted to pH 3.1 with solid citric acid. This procedure is carried out with constant stirring by means of a mechanical stirrer.

4 A small quantity of powdered pepsin 1 10,000 is added and the solution is stirred until all of the pepsin is dissolved. The pH value of the solution rises to 3.2. The quantity of pepsin chosen is such that the value of the ratio of the amount of pepsin to the quantity of protein (pepsin/total solids) is less than 1/10.

5 The solution pH 3.2 is kept for one hour at room temperature 20 to 25° C.

6 After one hour has elapsed, the pH value of the solution has risen to 3.23 to 3.26. A maximum rise of 0.06 pH is permissible in this procedure otherwise extensive digestion of horse serum proteins takes place with the production of substances commonly associated with the enzymatic decomposition of protein. The solution is adjusted to pH 4.2 with rapid stirring, using 30 per cent sodium hydroxide solution. A white precipitate becomes evident. The precipitate is a complex aggregate of denatured protein which was associated with the antitoxic pseudoglobulin fraction of the plasma<sup>7</sup> combined with pepsin.

7 After one hour has elapsed, 14 per cent by weight of ammonium sulfate is added and the solution is heated as rapidly as possible by means of steam to 58° C in order to coagulate the unwanted proteins. The solution is kept at 58° C for one hour. It is then permitted to cool.

8 After the precipitate has been allowed to settle for about forty eight hours, the supernatant solution is siphoned off and filtered through a Büchner filter funnel covered with a layer of Super-cel. After filtering all of the supernatant solution, the coagulum is then passed through the same filter. Both the supernatant solution and the coagulum are filtered by gravity.

9 The filtrate with a pH of about 4.1 is adjusted to pH 7.0 with 30 per cent sodium hydroxide solution which is added with constant stirring. Twenty per cent by weight of ammonium sulfate is then added to the solution and the antitoxic globulins are precipitated. The solution is then permitted to stand for one-half hour and is filtered through hardened filter paper.

10 The precipitate is pressed dry and placed in a cellophane bag. The precipitate is dialyzed until the sulfate ion is negligible. After dialysis is complete, the pH of the solution is about 6.3. The solution of antitoxin is brought to the neutral point by the addition of normal sodium hydroxide solution. One per cent by weight of sodium chloride is added to the antitoxin as well as 0.05 per cent Merthiolate and 0.35 per cent phenol. The potency of the antitoxin solution is then tested by flocculation or by animal protection tests.

Chemically pure reagents were used throughout the procedure. Various commercial brands of pepsin were employed of digestive potencies ranging from 1/3,000 to 1/10,000. Hydrogen-ion values of the antitoxin solutions were determined with an Electron-ray pH meter in conjunction with a MacInnes-Bellier condenser type of glass electrode. This apparatus has been adequately described in a previous paper.<sup>23</sup>

*Sensitivity Tests*—Sensitivity tests were performed on rabbits previously immunized to determine whether there were any horse serum reactive protein

in the antitoxin solutions under test. The sensitizing fluid consisted of an alum precipitated normal horse serum prepared by adding an equal volume of a 4.0 per cent solution of aluminum potassium sulfate to sterile normal horse serum. The precipitate is allowed to settle for twenty-four hours. The supernatant solution which contains but a trace of protein is discarded and replaced with a volume of physiologic salt solution equal to the original volume of normal horse serum.

Rabbits were immunized before being used for sensitivity tests by intraperitoneal injections with 5 ml. doses of alum precipitated normal horse serum once a week for five consecutive weeks. It was observed that the intradermal injection of normal horse serum containing as little as 0.02 mg. of protein nitrogen produced a skin reaction in a sensitized rabbit. The shaven back of a white rabbit was marked off into squares of approximately 9 sq. cm. with a blue marking pencil. One-tenth milliliter of the test fluid was injected intradermally into each of the squares. The test fluids consisted of horse plasma antitoxic globulins recovered by fractionation of plasma with ammonium sulfate and antitoxic pseudoglobulins obtained by means of the enzyme process previously described. All of the test fluids were diluted with 5 volumes of physiologic salt solution before injection into a rabbit. In practice it was found necessary to dilute the plasmas and antitoxic globulins not treated by the enzyme process with at least 5 volumes of saline solution in order to contain the skin reactions within the limits of the squares marked off on the back of the rabbit. Skin reactions were observed for a period of seventy-two hours; then the rabbits were discarded. The rabbits thus discarded were not employed in any subsequent test.

## RESULTS

The results of the purification of diphtheria antitoxin by the use of pepsin are shown in Table I. The second column gives the material which was treated by the enzyme process. The next column gives the pH value at which the enzyme treatment was carried out. This is followed by the potency of the pepsin which was added to the diluted antitoxin solution. The next column gives the potency of the antitoxin before and after enzyme treatment and concentration in units per milliliter obtained by the Ramon flocculation test. The remaining columns of the table are devoted to data on skin sensitivity tests before and after pepsin purification. The rabbit skin reactions are denoted as follows: 1 plus erythema, 2 plus erythema and edema, 3 plus erythema, edema, and necrosis,  $\frac{1}{2}$  plus a mild reaction. No visible skin reaction is recorded as zero.

Table I indicates that the first four lots of enzyme purified antitoxin produced slight skin reactions; especially the first lot which was treated at pH 4.0 with 1:3,000 pepsin. Moreover, there was a twofold increase in the antitoxic titer as shown by flocculation tests. Beginning with the fifth lot the potency increased about three and one-half times that of the untreated antitoxin. Treating the diluted antitoxin with 1:10,000 pepsin at pH 3.2 for one hour at room temperature, according to the method described previously, resulted in an antitoxic globulin which gave no skin reaction. An increase in the pH value at which

enzyme purification was carried out resulted in an antitoxin which produced an increased skin reaction. From an examination of Table I it is evident that the untreated antitoxic globulins in all cases gave more severe reactions than the enzyme purified globulins. Table I also shows that purification at pH 3.2 with 1:10,000 pepsin gave a more potent product than purification and concentration at the same pH value with 1:3,000 pepsin. Finally, while the untreated

TABLE I THE PURIFICATION OF DIPHTHERIA ANTITOXIN WITH PEPSIN

LOT	MATERIAL	pH	PEPSIN	POTENCY (UNITS/ML)		RABBIT SKIN TEST					
				BEFORE PEPSIN TREAT- MENT	AFTER PEPSIN PURIFI- CATION & CON- CENTRA- TION	BEFORE PEPSIN TREATMENT (HR.)			AFTER PEPSIN PURIFICATION (HR.)		
						24	48	72	24	48	72
1	RAG	4.00	1:3,000	1228	2060	++	+++	+++	++	++	++
2	RAG	3.20	1:3,000	1228	2332	++	+++	+++	+	+	+
3	RAG	3.20	1:3,000	1228	2576	++	+++	+++	+	+	+
4	RAG	3.25	1:3,000	1228	2511	++	+++	+++	+	+	+
5	RAG	3.19	1:10,000	1228	3180	++	+++	+++	-	-	-
6	RAG	3.23	1:10,000	1370	3300	+++	+++	+++	0	0	0
7	RAG	3.24	1:10,000	1370	4560	+++	+++	+++	0	0	0
8	RAG	3.22	1:10,000	1370	4560	+++	+++	+++	0	0	0
9	RAG	3.25	1:10,000	1370	4560	+++	+++	+++	0	0	0
10	RAG	3.22	1:10,000	1370	4880	+++	+++	+++	0	0	0
11	RAG	3.22	1:10,000	1370	4880	+++	+++	+++	0	0	0
12	RAG	3.23	1:10,000	1370	4800	+++	+++	+++	0	0	0
13	RAG	3.44	1:10,000	1370	4400	+++	+++	+++	+	+	+
14	RAG	3.57	1:10,000	1370	4660	+++	+++	+++	+	+	+
15	RAG	3.80	1:10,000	1370	4400	+++	+++	+++	++	++	++
16	RAG	4.00	1:10,000	1370	4400	+++	+++	+++	++	++	++

RAG Reconcentrated antitoxic globulin

+ Erythema ++ erythema and edema +++ erythema edema and necrosis - mild reaction  
visible reaction

antitoxin gave skin reactions which became intensified as time increased, the intensity of the skin reactions resulting from some of the pepsin-treated antitoxins diminished.

Table II shows the results of enzyme purification of tetanus antitoxin. The data are arranged in the same manner as in Table I. The potency of the antitoxins is expressed in units per milliliter as obtained by animal protection tests. An examination of Table II shows that approximately the same results were obtained as with diphtheria antitoxic globulins. It is to be noted that tetanus plasma gave the most severe skin reaction. Fractionation of plasma with ammonium sulfate reduced the intensity of the skin reaction somewhat. However the enzyme-treated globulins almost invariably gave no visible skin reaction. The results obtained with the last three lots shown in Table II indicate that antitoxic sera may be purified by treatment with pepsin in accordance with the procedure outlined in this paper, regardless of whether the initial material is immune horse plasma or antitoxic pseudoglobulins recovered from ammonium sulfate fractionation.

Table III contains the results of heating diluted tetanus antitoxic plasma and diluted normal horse serum to 58° C. at pH 4.2 in the absence of salt and

TABLE II PURIFICATION OF TETANUS ANTITOXIN WITH PEPSIN

MATERIAL	pH	PEPSIN	POTENCY (UNITS/ML)		RABBIT SKIN TEST					
			BEFORE PEPSIN PURIFI- CATION	AFTER PEPSIN PURIFI- CATION (CENTRA- TION)	BEFORE PEPSIN PURIFICATION (HR)			AFTER PEPSIN PURIFICATION (HR)		
					24	48	72	24	48	72
RAG	4.00	1 3 000	2000	2300	+++	+++	+++	+	++	+
RAG	3.98	1 3,000	2000	2200	+++	+++	+++	+	+	+
Oxalated plasma	3.20	1 10 000	3000	6500	+++	+++	+++	0	0	0
Oxalated plasma	3.20	1 10,000	3000	5000	+++	+++	+++	0	0	0
Oxalated plasma	3.21	1 10 000	2000	4200	+++	+++	+++	0	0	0
Oxalated plasma	3.20	1 10 000	2700	3500	+++	+++	+++	0	0	0
RAG	3.26	1 10 000	1450	2000	+++	+++	+++	0	0	0
RAG	3.24	1 10 000	1200	2700	+++	+++	+++	0	0	0
RAG	3.20	1 10,000	2000	2000	+++	+++	+++	0	0	0
Citrated plasma	3.25	1 10 000	6500	1600	+++	+++	+++	0	0	0
Citrated plasma	3.20	1 10,000	250	2000	+++	+++	+++	+	0	0
Concentrated antitoxic globulin	2.20	1 10 000	800	1500	+++	+++	+++	+	++	+
RAG	3.20	1 10 000	1200	1800	+++	+++	+++	0	0	0

RAG Reconcentrated antitoxic globulin

+ Erythema +++ erythema edema and necrosis + mild reaction 0 no visible reaction

also in the presence of 5 per cent sodium chloride Tetanus plasma was first treated for one hour at room temperature with 1 10 000 pepsin at pH 3.20 After one hour had elapsed the pH of the diluted plasma increased to 3.23 The pH value of the solution was adjusted to 4.20 After the solution had

TABLE III THE EFFECT OF TEMPERATURE AND SALT ON THE PURIFICATION OF TETANUS PLASMA AND NORMAL HORSE SERUM

MATERIAL	SKIN TEST (HR)			HEATED TO 58° C AT pH 4.2			HEATED TO 58° C AT pH 4.2 5% NaCl			PEPSIN PROTEIN PRECIPITATE		
	SKIN TEST (HR)			SKIN TEST (HR)			SKIN TEST (HR)			SKIN TEST (HR)		
	24	48	72	24	48	72	24	48	72	24	48	72
sted tetanus la ma	+++	+++	+++	+	+	+	0	0	0	0	0	0
sted normal one serum	+++	+++	+++	+	+	0	0	0	0	0	0	0

+ Erythema +++ erythema, edema and necrosis + mild reaction 0 no visible reaction

stood for one hour a copious precipitate was evident The diluted plasma was then divided into three aliquot portions The first was centrifuged and the precipitate was separated from the remainder of the solution The precipitate was then suspended in physiologic saline solution and adjusted to pH 6.95 The second portion of diluted plasma was heated to 58° C for one hour and placed in the refrigerator overnight The next day the precipitate was removed by filtration and the filtrate adjusted from pH 4.17 to pH 6.96 This procedure

was repeated with the third portion of diluted plasma with the exception that before heating the solution to  $58^{\circ}\text{C}$ , 5 per cent by weight of dry sodium chloride was added. These procedures were also carried out using normal horse serum which had been treated with 1:10,000 pepsin in the manner outlined in this paper. The protein nitrogen content of the solutions was determined by the micro-Kjeldahl procedure of Parnas and Wagner.<sup>29</sup> Skin tests were made with solutions of equal nitrogen value. One-tenth milliliter of the test fluids contained 0.2 mg of protein nitrogen.

An examination of the data in Table III shows that both the diluted normal horse serum and the diluted tetanus plasma having a protein nitrogen content of 2.0 mg per milliliter gave the most severe reactions. This fact has already been mentioned in connection with the skin test data shown in Table II. Less severe reactions were observed with diluted normal horse serum and with the diluted tetanus plasma which had been heated to  $58^{\circ}\text{C}$  in the absence of salt. From an examination of the fourth column of Table III it is evident that there was no visible reaction resulting from the intradermal injection of the diluted tetanus plasma or the diluted normal horse serum which had been heated to  $58^{\circ}\text{C}$  in the presence of 5 per cent sodium chloride. The unheated pepsin-protein precipitates recovered from the diluted tetanus plasma and the diluted normal horse serum at pH 4.20 gave no visible skin reactions.

Further studies indicated that all but a trace of heat coagulable ( $100^{\circ}\text{C}$ ) proteins were absent from the solutions of normal horse serum and tetanus plasma which had been heated to  $58^{\circ}\text{C}$  at pH 4.20 in the presence of 5 per cent sodium chloride. Moreover, 3.3 per cent of heat coagulable protein still remained in the solution of tetanus plasma which had been heated to  $58^{\circ}\text{C}$  in the absence of salt. Also 2.2 per cent of heat coagulable protein was still present in the diluted normal horse serum which had been heated to  $58^{\circ}\text{C}$  in the absence of 5 per cent sodium chloride. These results indicate that the skin reacting substances which are present in tetanus plasma and in diluted normal horse serum vary with the amount of heat coagulable protein which remains in the solution after the enzyme process is completed.

The lack of any visible skin reaction from the injection of the pepsin protein precipitate which occurs at pH 4.2 indicates that this precipitate consists of a complex aggregate of denatured protein which has become incapable of eliciting any response from a sensitized rabbit. Furthermore this denatured protein is associated with pepsin which has been added to the diluted plasma or serum. Tests show that the added pepsin is absent from the diluted plasma or serum at pH 4.2 after heating to  $58^{\circ}\text{C}$  in the presence of 5 per cent sodium chloride or 1.4 per cent ammonium sulfate. By the use of a modification of the hemoglobin test devised by Anson<sup>30</sup> the preceding solutions as well as the enzyme-treated globulins mentioned in Tables I and II were found to be free of any peptic action after the enzyme procedure was completed.

#### DISCUSSION

The procedure described in this paper for the purification of antitoxic horse plasma and antitoxic pseudoglobulins has been tried using other types of normal



and immune animal sera. I have observed that if bovine or goat globulins are treated with pepsin and the diluted globulin solution is adjusted to pH 4.2 a copious white precipitate appears in a short time. This observation has been made with normal horse serum as shown in Table III. Other investigators<sup>6-31</sup> besides Pope have made similar observations.

It has been known for some time that crystalline proteins such as edestin<sup>32</sup> are capable of removing all but traces of pepsin from a solution at pH 4.0. Waldschmidt Leitz and Kofran<sup>33</sup> have observed a similar phenomenon with melon seed globulin. Northrop<sup>3</sup> has demonstrated that the pepsin-edestin precipitate may be dissociated under suitable conditions so that the original activity of the pepsin is restored. Neurath and colleagues<sup>34</sup> have discussed the role of enzymes in protein denaturation. The precipitate at pH 4.2 consists of serum proteins which have been denatured by proteolytic enzymes. In the case of horse serum proteins which have been denatured with pepsin at pH 4.2, such proteins are incapable of causing a skin reaction in rabbits sensitive to horse serum as shown by the data in the fifth column of Table III.

This discussion would be incomplete without a brief statement concerning the clinical use of enzyme purified antitoxins. Out of twenty-six recorded cases of patients treated with enzyme purified tetanus antitoxin, twenty patients recovered completely, with only one developing serum sickness and one having a mild reaction to the antitoxin. The first patient had mild serum sickness which occurred about eight days following the first injection. It was learned subsequently that the patient was horse serum sensitive and had been given the first dose of tetanus antitoxin obtained by ammonium sulfate fractionation. The other patient received a total of 3,580,000 units of antitoxin in a period of nine days. This patient also recovered despite a mild reaction to the antitoxin. All the familiar routes of injection were employed in all the patients. Seven of the patients had clinical histories of bronchial asthma and horse serum allergy. The age of the patients treated with the antitoxin ranged from four to sixty-five years.

In another series of clinical tests, prophylactic injections of enzyme purified tetanus antitoxin were given to fifty patients in an accident ward of a hospital. All of the patients had clinical histories of asthma, hay fever or horse serum sensitivity. No immediate or delayed reactions were observed.

Enzyme purified diphtheria antitoxin has been used in two patients with diphtheria who were known to be sensitive to horse serum. One of the patients was a child of four, the other was an adult about sixty years old. Both patients recovered. Neither of the patients exhibited any signs of serum sickness.

Both the enzyme purified tetanus and diphtheria antitoxin have been employed on other asthmatic or horse serum sensitive patients with excellent results.

#### SUMMARY

A method has been described for the purification of diphtheria and tetanus antitoxin by the use of pepsin. The results of the purification of antitoxic horse

plasma or antitoxic globulins by pepsin indicate that the enzyme treated globulins give no skin reaction in rabbits which have been immunized to the proteins of normal horse serum. Some clinical results of the use of enzyme purified antitoxins are also described.

The author takes this opportunity to thank Mr C K Greenwald, Mr A. Mackey, and Mr M. Burger for their assistance in performing the sensitivity tests and for the determination of the potencies of the samples of tetanus antitoxin, and also Mrs E Crozman for obtaining the potencies of the samples of diphtheria antitoxin by the Ramon flocculation test.

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# ANTIRETICULAR CYTOTOXIC SERUM IN THE TREATMENT OF ARTHRITIS

## OBSERVATIONS ON FORTY-FOUR CASES

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ANTIRETICULAR cytotoxic serum (A.C.S.) has been described as an effective remedy in different chronic conditions including several types of rheumatism. The present study was planned to test its effectiveness in the treatment of two types of chronic joint disease—rheumatoid arthritis and degenerative joint disease.

Bordet originally observed that antibodies were formed in animals under proper conditions after injection of human material such as leucocytes, liver or kidney cells. The serum of such immunized animals produced varying reactions when injected into human beings. Large doses caused toxic effects while small doses sometimes produced beneficial stimulating effects.

Bogomolets<sup>1</sup> became interested in this phenomenon in 1929. After considerable study he concluded that connective tissue was the most important element in antibody production. He therefore attempted to develop antigens by injecting material from the human reticulo-endothelial system. His report in 1943 that his serum was effective in the treatment of rheumatism stimulated interest in America and since that time several reports have appeared. The theoretic considerations have been reviewed by Straus<sup>2</sup>.

The material\* used in this study was the same as that supplied for other studies (Rogoff and co-workers<sup>3</sup>). Two types of material were provided. One contained antireticular cytotoxic serum in rabbit serum and the other contained only untreated rabbit serum. The identity of these sera was unknown to us until the study was completed. The antireticular cytotoxic serum was prepared by injecting into rabbits and goats a saline extract of spleen and bone marrow secured within ten hours of death from persons under 40 years of age, apparently in good health, who died from sudden injury. The saline extract was injected intravenously into rabbits or goats. The immunized animals were bled and the sera were dehydrated and stored until needed. When used it was diluted to a 10 per cent solution in normal saline.

Bogomolets's method of administration was followed closely. Injections of the antireticular cytotoxic serum and the control serum were given subcutaneously. The first dose was 0.5 c.c. followed at intervals of three to five days with a second dose of 1.0 c.c. and a third dose of 1.5 cubic centimeters. Three doses constituted a series. A second and third series were given at six week intervals. The three series were considered a complete treatment.

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The patients were selected from the Out Patient Department of Cleveland City Hospital. All were ambulatory and many had been under observation for months to years. The diagnoses of rheumatoid arthritis and of degenerative joint disease depended upon the usual clinical criteria and were supported by laboratory and radiologic evidence. Patients in each category were selected alternately for the antireticular cytotoxic serum and the control serum.

## RESULTS

Table I shows the results in rheumatoid arthritis. Of the twenty patients with rheumatoid arthritis, seven were subjected to three complete series of injections with antireticular cytotoxic serum and seven had three complete series of injections with control serum. In addition to these fourteen patients there were eight patients who received less than the three complete series, five had

TABLE I RHEUMATOID ARTHRITIS

	NUM BER	AVERAGE AGE (YR.)	AVERAGE DURA TION (YR.)	OBJECTIVE RESULTS			SUBJECTIVE RESULTS		
				BETTER	SAME	WORSE	BETTER	SAME	WORSE
A. C. S.									
Completed series	7	52	5	1	5	1	1	5	1
Total cases	12	49	5	2	8	2	2	7	3
Total (%)				16	67	16	16	58	25
Control Serum									
Completed series	7	55	9	1	5	1	1	5	1
Total cases	8	56	8	1	6	1	1	6	1
Total (%)				12	75	12	12	75	12

antireticular cytotoxic serum and one the control serum. Table II shows twenty four patients with degenerative joint disease six received three complete series of injections with antireticular cytotoxic serum and ten received three complete series with control serum. As with the patients with rheumatoid arthritis there

TABLE II DEGENERATIVE JOINT DISEASE

	NUM BER	AVERAGE AGE (YR.)	AVERAGE DURA TION (YR.)	OBJECTIVE RESULTS			SUBJECTIVE RESULTS		
				BETTER	SAME	WORSE	BETTER	SAME	WORSE
A. C. S.									
Completed series	6	56	9	0	6	0	4	2	0
Total cases	14	58	10	1	13	0	6	8	0
Total (%)				7	93		43	57	
Control serum									
Completed series	10	60	7	2	8	0	5	5	0
Total cases	10	60	7	2	8	0	5	5	0
Total (%)				20	80		50	50	

were eight additional patients who received one or two series of antireticular cytotoxic serum but did not finish the three complete series.

Reactions were more frequent after injections with antireticular cytotoxic serum than with control sera. A general reaction including mild fever, malaise

and nausea was observed four times after antirreticular cytotoxic serum and once after control serum. Local reactions with tenderness, swelling, and redness at the site of injection occurred after eighteen injections with antirreticular cytotoxic serum and four times after injections of control sera.

The tables list both objective and subjective results. Objective improvement meant a decrease in swelling, increase of motion, or loss of tenderness. The subjective results were based upon the patients' own estimation of their conditions. The subjective results followed very closely the objective results in most instances. In the patients with rheumatoid arthritis receiving antirreticular cytotoxic serum two were better, eight were unchanged, and two were worse objectively, compared with one better, seven unchanged, and three worse subjectively. Of the fourteen patients with rheumatoid arthritis, seven of whom received three complete series of antirreticular cytotoxic serum and seven of whom received three complete series of control serum, the tabulations of objective and subjective results were identical.

Subjective improvement was more apparent than objective improvement in the patients with degenerative joint disease. Of fourteen patients receiving antirreticular cytotoxic serum, only one was listed as having objective improvement while six thought they had been benefited. It seemed significant that of the ten patients receiving control serum, two were listed as having objective improvement and five thought they had been helped.

No significant difference was observed between the use of antirreticular cytotoxic serum and control serum. Every patient was seen over a period of at least six months and many were under observation for over a year. Any critical observer at all familiar with the clinical vagaries of rheumatoid arthritis and of degenerative joint disease recognizes the frequency with which patients with these chronic diseases describe variations in the degrees of their symptoms, especially when they are observed for a long period of time and under changing therapeutic regimens. Such mild variations, occurring as they often do spontaneously, are of no significance in judging therapeutic effectiveness unless they are sustained. The authors feel that the variation in patients' symptoms recorded in this study are not an indication of specific therapeutic effect for antirreticular cytotoxic serum.

The results obtained in this study are in essential agreement with other recent reports. Bach<sup>4</sup> treated forty-eight patients, thirty-five had rheumatoid arthritis and the others had rheumatic fever, ankylosing spondylitis, gonorrheal arthritis, osteoarthritis, and nonarticular rheumatism. Bach noted clinical improvement in fourteen, in seven it was definite and in seven only slight. The patients with definite improvement included three with rheumatoid arthritis, one with rheumatic fever, one with gonorrheal arthritis, one with ankylosing spondylitis, and one with muscular rheumatism. In two of the three cases of rheumatoid arthritis the benefit observed might well have been due to bed rest for over a month in a hospital. Two months after discharge both patients suffered a relapse. The author discusses the difficulty in evaluating the influence of the serum in producing the benefits observed.

Rogoff and associates<sup>3</sup> treated twenty nine patients with rheumatoid arthritis and ankylosing spondylitis with antireticular cytotoxic serum and fourteen patients with rabbit serum. They noted that 10 per cent of the patients had symptomatic improvement, 10 per cent had objective improvement, 55 per cent were unchanged, and 24 per cent were worse. Results of the same order were observed with the use of control rabbit serum. They concluded that antireticular cytotoxic serum therapy produced no dependable benefit in patients with rheumatoid arthritis.

#### COMMENT

The effects observed from the treatment of twenty patients with rheumatoid arthritis and of twenty four patients with degenerative joint disease were so slight and inconclusive that further investigation did not seem to be justified. Despite the small number of patients treated and the negative results obtained, this report is in complete agreement with recent similar observations on this subject and is presented to place these results on record as part of the observed experience with this form of treatment.

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# THE THIOCYANATE CONTENT OF SALIVA IN NORMAL AND HYPERTENSIVE SUBJECTS BEFORE AND AFTER INGESTION OF THE DRUG

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EARLY studies, as surveyed by Lickint,<sup>1</sup> are inconsistent regarding the thiocyanate content of human saliva in health. More recently Reissner<sup>2</sup> found a normal range of 2.4 to 20.0 mg per 100 ml with an average of 10.0 mg per cent thiocyanate (SCN). Claims regarding salivary SCN changes in certain pathologic conditions<sup>3</sup> remained unconfirmed. Similar difficulties have been encountered with other salivary constituents. With special reference to calcium and phosphorus, Becks and Wainwright<sup>4</sup> criticized older reports because the method of obtaining specimens was not stated, or was unsatisfactory, and because of disregard for the rate of flow. They suggest that the collection of resting saliva gives uniform and comparable results. Such criticism also applies to most reported thiocyanate studies.

In 1925 Nichols<sup>5</sup> found that SCN concentration of saliva is considerably raised after SCN administration. Tastaldi,<sup>6</sup> in hypertensive patients treated with potassium thiocyanate (KSCN), found that the saliva:serum ratio decreased with increasing serum levels. He did not report on natural salivary SCN. The SCN content of other body fluids was repeatedly studied in thiocyanate treated hypertensive patients and as a means of measuring extracellular fluid volume.

The aim of the present study was to obtain more reliable and uniform information than has hitherto been available regarding natural and post-ingestion salivary thiocyanate levels by using a standardized method of collection, and by relating values to the rate of flow.

## MATERIAL AND METHOD

Three groups of salivary SCN determinations were made: (a) Determination of the natural level in each of thirty-five healthy subjects. (b) Three determinations of the pre-ingestion level at short intervals in thirty-five patients with raised blood pressure. (c) Determination of the level at intervals of one to two weeks, for periods varying from four weeks to several months, in thirty patients with raised blood pressure during treatment with thiocyanate salts. The drug was administered as described in a previous paper. In the second and third group the serum level also was determined. One hundred and forty determinations were made before and one hundred and seventy-six after administration of KSCN.

The subject was instructed to have nothing to eat or drink on the morning of the test, not to brush his teeth, and not to smoke. The specimen was collected between 9 and 10 A.M. the subject bent his head over a funnel placed on a graduated centrifuge tube and the saliva was allowed to flow freely with exclusion of movements of the jaw, spitting, and so forth. The time of collection was ten to thirty minutes. The specimen was then centrifuged and 1 cc. of the clear supernatant fluid was used for the determination of thiocyanate by the method of Schreiber<sup>7</sup> as modified by Crundall and Anderson.<sup>8</sup>

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*Naturally Occurring Salivary Thiocyanate —*

*Concentration of SCN in Milligrams Per Cent* The arithmetic mean in thirty five normal subjects was  $13.4 \pm 1.1$  mg per cent. The lowest and highest observed values were 3.1 and 27.5 mg per cent. No significant difference was demonstrable between different age groups and the sexes.

The arithmetic mean for nonsmokers was  $11.7 \pm 1.0$  mg per cent. The range varied from 3.1 to 26.5 mg per cent. The arithmetic mean for smokers was  $17.5 \pm 2.1$  mg per cent. The range varied from 8.1 to 27.5 mg per cent. The means differed considerably, the ranges however overlapped to such an extent that no significantly different range for nonsmokers and smokers could be established. Patients were arbitrarily divided into three groups according to the rate of flow (under 10, 10 to 20 and above 20 cc per hour respectively).

TABLE I RELATION OF SCN CONTENT OF SALIVA OF HEALTHY AND HYPERTENSIVE SUBJECTS TO THE RATE OF FLOW AND SMOKING

GROUP	MG PER CENT						MG PER HOUR					
	MEAN		$\sigma$		$\sigma M$		MEAN		$\sigma$		$\sigma M$	
	N	H	N	H	N	H	N	H	N	H	N	H
Total	13.4	14.7	6.3	6.0	1.1	1.0	2.34	1.93	1.23	1.15	0.21	0.20
Nonsmokers												
Total	11.7	14.3	5.5	6.2	1.0	1.3	2.25	1.81	0.98	1.02	0.16	0.19
RF under 10 cc/hr	17.3	16.5	5.5	5.6	2.0	1.3	1.49	1.49	0.37	0.28	0.13	0.16
RF over 15 cc/hr	9.3	9.5	3.7	4.0	0.9	1.3	2.38	2.19	0.82	1.65	0.19	0.55
Smokers												
Total	17.5	16.2	6.3	4.7	2.1	1.7	3.18	2.40	1.64	1.57	0.55	0.59
RF under 15 cc/hr	22.2	19.2	3.8	2.9	2.2	1.4	2.30	1.46	0.30	0.45	0.17	0.22
RF over 15 cc/hr	15.2	14.1	6.0	3.3	2.5	1.9	3.93	3.66	1.76	1.63	0.72	0.94

RF Rate of flow. N normal group. H hypertensive group.  $\sigma$  standard deviation of the mean.  $\sigma M$  standard error of the mean.

Comparisons of the arithmetic means of the corresponding milligram per cent values (22.5, 14.3, and 9.8) suggest an inverse relationship between rate of flow and SCN milligram per cent concentration. The correlation coefficient,  $r = -0.65 \pm 0.10$ , is significant. In order to show that the high values encountered in the low rate of flow group were not due to smoking, nonsmokers and smokers were grouped separately into two groups of higher and lower rate of flow (below and above 15 cc per hour). Table I shows that the inverse relationship of rate of flow and concentration was present irrespective of smoking. There was practically no overlapping of concentrations of nonsmokers in the low and medium rate of flow groups, the highest concentration in the latter being 15.5 mg per cent, the lowest in the former being 15.1 mg per cent.

The arithmetic mean in thirty five hypertensive patients was  $14.7 \pm 1.0$  mg per cent. The lowest and highest observed values were 4.7 and 31.1 mg per cent. This range practically coincides with the one found in normal subjects and the arithmetic means do not differ significantly. Figures in Table I also demonstrate

the inverse relationship between rate of flow and milligrams per cent concentration of SCN in hypertensive patients

*SCN Content of Saliva Expressed as Milligrams Per Hour* Normal values in nonsmokers and smokers and in hypertensive individuals are shown in Table I. It appears that there is a direct relationship between rate of flow and milligram per hour values

*Concentration of SCN After Ingestion of KSCN*—Natural SCN value means the SCN content of saliva without ingestion of SCN salts. Additional value expresses the amount of ingested SCN diffusing into the saliva. Total value is the actual value observed after ingestion and is made up of the sum of the natural and additional values. The range of concentration of SCN after ingestion of varying doses of KSCN was 16.8 to 36.9 mg per cent. The range of additional values after ingestion fell between 2.0 and 21.7 mg per cent. In general a rise or fall followed an increase or decrease in the dose, the concentration remaining constant on a given dose. Often however after a marked initial rise further increase was only slight on raising the dose. In one subject the additional value of 17 mg per cent rose to only 19.6 on raising the dose from the

TABLE II. MEAN AND RANGE MILLIGRAM PER CENT CONCENTRATIONS ADDED TO NATURAL MILLIGRAM PER CENT CONCENTRATIONS OF SALIVA AFTER INGESTION OF KSCN, AT VARYING SERUM LEVELS

SALIVA WITH NATURAL VALUE	SERUM (MG %)					
	2.4	4.6	6.8	8.10	10.12	12.14
Under 9 mg %						
Mean	—	14.7	16.9	14.4	20.8	19.7
Range	—	10.7-21.1	11.6-23.4	11.6-17.6	20.5-21.1	17.6-21.1
9-19 mg %						
Mean	9.7	9.7	12.3	12.5	12.3	12.3
Range	6.4-14.7	5.1-16.3	7.2-17.5	8.7-18.0	9.6-15.7	11.7-17.6
Over 19 mg %						
Mean	8.3	9.8	9.9	11.6	15.6	—
Range	—	2.0-15.1	5.8-12.9	11.0-15.0	14.5-17.2	—

initial 0.4 to 2 grams. In another subject the additional value of 15.2 mg per cent with a dose of 0.6 Gm rose to 17.1 with 1.2 grams. The average concentration accompanying doses ranging from 0.13 to 1.2 Gm rose from 25.2 to 31.2 mg per cent. Ranges overlapped widely and were indistinguishable for different doses. The additional value was higher in the low than in the two upper natural value groups, with only slight difference between the latter two (Table II). There was a slight rise in total average saliva concentration, accompanying increased serum values. The rise was less pronounced than in serum. Ranges accompanying divergent serum levels overlapped. The relation of serum levels and additional values was similar (Table II). In most cases the additional concentration in saliva was two to three times higher than that in the serum, the saliva:serum ratio ranging from 7 to 12. Only in one case out of thirty was the ratio definitely lower than 1. The range of milligrams per hour values after ingestion was 1.26 to 10.10, the range of additional concentration, 0.43 to 6.05. There was much greater irregularity in variation of milligrams per hour than in milligram per cent values, owing to fluctuation of the rate of flow.

## DISCUSSION

The normal range of milligram per cent concentrations found in the present series is wider than that in most previous publications the average being somewhat higher. This is chiefly due to the different method of collection.

Becks and Wainwright<sup>4</sup> pointed out that most constituents of human saliva show increased concentration with a higher rate of flow. This applies both to milligram per cent and milligram per hour values. Two exceptions were found in human resting saliva, the calcium and phosphorus concentrations expressed in milligrams per cent are inversely proportional to the rate of flow while milligram per hour values show an increase with a higher rate of flow. According to the present study, SCN shows this same type of exceptional behavior.

Past attempts to establish a normal salivary SCN range and to relate abnormal values to certain pathologic states<sup>1</sup> were prevented by wide overlapping of ranges.<sup>10</sup> It seems that unless the normal range can be narrowed with reference to certain factors such as rate of flow or smoking SCN determinations will be of no diagnostic value. Lickint<sup>1</sup> attempted to reduce the range by classification according to smoking. The present findings suggest that this alone is not enough. By employing a standard method of collection and by grouping results according to rate of flow and smoking the range has been narrowed down. Future research must decide whether this will be sufficient to define abnormal values.

Caviness, Bell, and Satterfield<sup>11</sup> found an inverse relationship between the height of blood pressure and naturally occurring serum thiocyanate. They considered thioevanite a true natural depressor substance. This was denied by others.<sup>12-13</sup> Findings in saliva also show no difference in SCN concentrations between normal and hypertensive patients.

Lack of constant correlation between dosage and salivary SCN also between serum and salivary SCN levels renders salivary SCN determinations unsuitable for the control of SCN therapy in hypertension. The tendency to inverse relationship between natural and additional concentration has a levelling effect on the total concentration observed obscuring changes due to change in dosage.

The higher the salivary SCN level at the time of administration of a given amount (regardless of whether this level is the patient's natural level or whether it was produced by previous administration of the drug) the smaller a proportion of the added amount will diffuse into the saliva. Since the height of dosage is limited by toxic effects, it could not be ascertained whether the salivary SCN can be raised to a point where further administration of the drug will not produce a further rise in salivary SCN. These observations together with the discrepancy found between saliva and serum concentrations seem to give experimental support in human beings to the suggestion put forward by Elkinton and Taftel,<sup>14</sup> based on animal experiments that the applicability of SCN distribution as a measure of extracellular body fluid volume is limited because of uneven distribution of SCN possibly owing to formation of depots. Saliva seems to constitute such a depot.

## SUMMARY

The thiocyanate content of human saliva has been determined using a standard method of collection and considering the rate of flow. Results are expressed in milligrams per cent and in milligrams per hour.

SCN differs from most salivary constituents in that milligram per cent values are inversely related to the rate of flow and directly related to milligram per hour values. Smoking is often accompanied by higher values. The significance of these factors in defining abnormal ranges is discussed.

Salivary SCN values in hypertensive subjects coincided with those in healthy subjects.

After administration of thiocyanate salts, the amount of SCN appearing in the saliva usually is greater than in serum. There is a tendency for inverse relationship between the amount of SCN appearing in the saliva and the concentration at the time of ingestion. These findings appear relevant in judging SCN distribution in body fluid.

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## LABORATORY METHODS

### A MODIFIED METHOD OF PREPARING THE J S B STAIN

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SINCE the introduction of blood staining, which is generally credited to Ehrlich,<sup>1</sup> many techniques have been developed, most of them modifications of the method devised by Romanowsky and published by him in 1891.<sup>2</sup> Of these the most widely adopted have been the methods of Leishman,<sup>3</sup> to Wright,<sup>11</sup> and Giemsa.<sup>4</sup> More recently the method recommended by Field<sup>5</sup> for staining thick films has been accepted widely. But a completely satisfactory stain for the demonstration of blood elements and blood parasites has yet to be discovered, as evidenced by the continual appearance of new processes. Some stains are expensive others may be cheap but do not keep well in all climates or they may be difficult to obtain. Still others, such as Giemsa's, stain too slowly.

For these reasons the appearance of the J S B stain (Smgh and Bhattacharya)<sup>10</sup> was welcomed by many laboratory workers. Its advantages have already been set forth by its originators and by one of us (R D M).<sup>7</sup> When properly prepared and used, it stains very quickly, giving excellent differentiation of the commonly encountered blood protozoa, it keeps well in aqueous solution for months and costs very little to make. The ingredients chiefly methylene blue and eosin, are available almost everywhere. It is a Romanowsky stain, in many respects similar to Field's.<sup>2</sup>

However, the method of preparation originally recommended has proved unreliable, and the work herein reported was initiated in an attempt to improve it.

The J S B staining process requires two solutions, one of which (Solution 1) is prepared by an acid oxidation of methylene blue and the other by making a 0.2 per cent solution of water soluble eosin in tap water. It is the preparation of Solution 1 which has proved troublesome. The directions originally prescribed for making the two solutions are as follows:

Solution I is made up from the following ingredients

Medicinal methylene blue	0.5 gm
Potassium dichromate	0.5 gm
Sulfuric acid (1 per cent)	30 c.c.
Water	500.0 c.c.

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Dissolve the methylene blue thoroughly in 500 cc of water. Add the 1 per cent sulfuric acid, mix thoroughly, and then add the chrome salt. A heavy amorphous purple colored precipitate of methylene blue chromate forms. Heat in an autoclave at a temperature of 100° to 109° C and a pressure of 0 to 5 pounds for three hours. At the end of this period the solution turns blue which indicates almost complete polychroming. If the color remains greenish, further heating for another hour or so is required. If the temperature is allowed to rise above 110° C, the oxidation of methylene blue may be carried too far and the solution will turn a violent purple.

When the solution has turned deep blue after three hours' boiling, allow it to cool at room temperature. Then add 10 cc of 1 per cent potassium or sodium hydroxide solution, drop by drop, very gradually while constantly shaking the flask. After the total amount of alkali has been added, transfer half of the contents of the flask into another of the same capacity and continue shaking for fifteen minutes more. Transfer the contents of the flasks into each other. In this way the precipitate will gradually get dissolved and the solution will turn deep blue with a violet iridescence. Leave it at room temperature for forty-eight hours, for the solution to mature, afterward filter through a soft filter paper. The solution will improve in staining qualities with age.

**Solution II** This is readily prepared by dissolving 1 gm of water soluble eosin in 500 cc of tap water. A freshly prepared eosin solution may not yield as satisfactory a stain as one which has turned deep red after some use.

After considerable experimentation we have found it best to filter out and collect the precipitate which forms after removing the mixture of methylene blue, dichromate, and acid from the boiling water bath (which we prefer to the autoclave) and cooling, and to dissolve it in 500 cc of M/20  $\text{Na}_2\text{HPO}_4$ . This solution should be allowed to mature for forty-eight hours, after which it is ready to use. It will keep well for some weeks, or even months, so that one may keep it on the laboratory table ready for use when needed. This fact makes the I S B stain one of the most convenient of all Romanowsky stains.

If it is desired to dry the precipitate, it should be done at room temperature. A vacuum desiccator will be found convenient. The dried stain appears to keep very well and may be redissolved as needed. A 0.1 per cent solution (100 mg to 100 cc of M/20  $\text{Na}_2\text{HPO}_4$ ) is easily made up and stains very satisfactorily.

Another method which may be used, but which we have found more cumbersome and less reliable, is to increase the amount of alkali added to neutralize the acid mixture after removal from the water bath or autoclave. Care must be used to keep the pH from passing 8.5, and the reaction becomes very sensitive when the hydrogen ion concentration reaches 7.5. A glass electrode pH meter is necessary for this purpose.

The yield of precipitate when the stain is prepared according to the formula given is about 0.56 gram. The use of several batches of methylene blue from many different manufacturers revealed no significant variations either in the amount or quality of the product, although it was at first suspected that such variation might be a possible reason for the frequent failure to obtain satisfactory results when the original directions were followed. The stain which comes out of solution in the form of very long, needle like crystals of a

deep blue color. In ethyl alcohol it has a solubility of  $2.2 \pm 0.05$  Gm per liter, and in distilled water the figure is almost the same being  $2.1 \pm 0.05$  Gm per liter.

Since Holmes and French found that the amount of dichromate used had a great deal to do with the character of the product several experiments were tried in which this reagent was reduced in quantity. When it was halved, it was found that the precipitate amounted to only about 0.51 Gm and when it was cut to 0.10 Gm (a reduction of 80 per cent) the yield was but 0.15 Gm (These and other data relating to these experiments are summarized in Table I.) In this last instance the character of the precipitate was also altered and it appeared that a large proportion of the methylene blue remained unoxidized.

Thus it appears, although some reduction of the dichromate may be made that any considerable change in this direction will reduce the yield of stain. In this connection it is necessary to point out that the experiments of Holmes and French are not wholly comparable to those we are reporting, since they used much more concentrated solutions of acid and methylene blue and a much shorter cooking time.

TABLE I. EFFECT OF REDUCTION IN AMOUNT OF DICHROMATE ON YIELD AND COMPOSITION OF STAIN

K <sub>2</sub> CrO <sub>7</sub> (GM)	H <sub>2</sub> SO <sub>4</sub> 1% (ML)	METHYLENE BLUE (GM)	YIELD (GM)	E <sub>640</sub> E <sub>670</sub>	STAIN		ABSORPTION MAXIMA (IN DISTILLED WATER) (Mμ)
					AZURE B (%)	METHYLENE BLUE (%)	
0.50	3.0	0.50	0.569	1.36	74.7	25.3	654 662
0.25	3.0	0.50	0.516	0.90	32.4	67.6	660 664
0.10	3.0	0.50	0.150	0.82	22.4	77.6	660 665

E is the extinction coefficient at the two wave lengths mentioned. The ratio change with changes in the proportion of methylene blue. See Holmes' for details of method and standard curve. The test were done with samples of stain in 50 per cent ethyl alcohol.

To get some information about the composition of the stain the precipitate was tested with the spectrophotometer, using samples dissolved in distilled water. The results indicated that it was a mixture of methylene blue and azure B in each case, the percentage of the latter being greatest (about 75 per cent) when the full amount of dichromate was used. These figures are also summarized in Table I.

Fig. 1 shows the absorption curve is determined with the spectrophotometer for the stain in aqueous solution when prepared with the amounts of ingredients specified in the original formula.

As a result of these determinations, it seemed worth while to try making up Solution I from methylene blue and commercial azure B using a mixture of approximately one part of the former to three of the latter and an amount of water sufficient to give a dye concentration equivalent to the laboratory prepared JSB stain. This solution was found to stain well although it did not seem quite equal to the latter. It also was found that the exact proportions of the two dyes were not very important; variations of perhaps 10 per cent either way did not change the quality of staining very much.

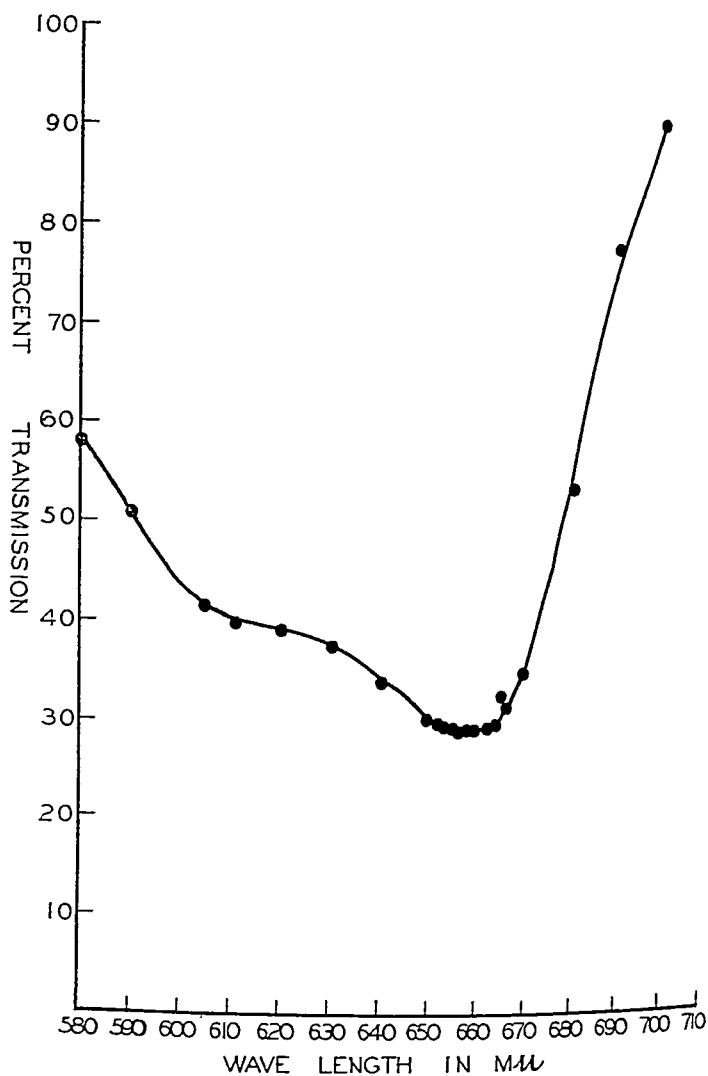


Fig 1—Absorption curve of JSB stain in H<sub>2</sub>O



In this regard it is worth pointing out that our results disagree with those of Holmes and French<sup>5</sup> who believed that azure B had little staining value, but they are in accord with those of Roe Lillie and Wilcox.<sup>6</sup> However, Holmes and French used a different technique.

In using J S B as a blood stain we have found it an advantage to buffer the wash water so that its initial pH (6.2 to 6.6) will be retained. For this purpose we use  $\text{Na HPO}_4$ ,  $7\text{H}_2\text{O}$  and  $\text{KH}_2\text{PO}_4$  in the proportions of 0.417 and 0.752 Gm, respectively, dissolved in two liters of distilled water.

Our experience indicates that this stain has very definite advantages over any of the other Romanowsky stains in common use. At current prices the materials for making it cost about one sixth as much as Giemsa powder and the disparity is considerably greater if Giemsa is purchased in stock solution. It also lasts much longer after being made up. It is as rapid as any stain other than Field's and is good for both thick and thin smears. Differentiation of the common protozoan blood parasites is excellent. We have had no opportunity to try it on blood spirochetes but it may prove useful for these also.

As a blood stain, it may be less satisfactory for routine use than Wright's and Giemsa's because small variations in the staining time may cause some differences in the appearance of the various blood elements. However, if these factors are controlled carefully, it should be of value for these purposes also.

In this laboratory it has proved useful for the staining of vaginal smears (from rats) and has saved time as well as given good cell differentiation.

Correspondence\* indicates that with a slight modification the J S B stain is very useful in the staining of dried films of milk and cream. However, the stain as modified in this way does not give satisfactory staining of blood parasites. (The chief change was the employment of a much lower pH 5.0 to 5.5 for Solution I. The smears themselves required fixing in a chloroform alcohol reagent for several minutes in order to remove the fat. After staining examination under the microscope was done with a suitable color filter to increase the contrast.)

#### SUMMARY

Several modifications of the original method of preparing the J S B stain are described. They involve filtering out the precipitate resulting from the acid oxidation of methylene blue and redissolving in M/20  $\text{Na HPO}_4$ , which serves both as a solvent and a buffer. The precipitate also can be dried and kept more or less indefinitely without deterioration.

Spectrophotometric tests show that the active staining agent is a mixture of azure B and methylene blue in a proportion of about 3 to 1. A solution of commercial azure B and medicinal methylene blue in these proportions was found to be a good substitute for the laboratory prepared product although apparently not quite equal to it.

\*With Dr. N. O. Gunderson of the Department of Public Health of Illinois.

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# CRYSTALLINE HEMIN SOLUTIONS AS PERMANENT STANDARDS FOR HEMOGLOBIN ESTIMATION

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IN A previous communication<sup>1</sup> solutions of crystalline hemin at pH 9.4 were proposed as standards for the colorimetric determination of hemoglobin. Such solutions can be reproducibly prepared from weighed quantities of crystalline hemin, and they have absorption spectra closely paralleling those of alkaline hematin from whole blood. With the proper filters and conversion factor the colorimetric determination of hemoglobin is thus referred to a readily available gravimetric standard. It was hoped that the standardization could be further simplified by the use of permanent standards kept in sealed colorimeter tubes and studies were begun to determine the long time stability of such solutions. They were found to have a useful life of about nine months, following which a progressive decrease in intensity became apparent.

Similar results have recently been reported by King<sup>2</sup> who investigated the stability of hemin solutions at pH 9.4 and found a sharp drop in intensity after nine months. The standard solutions used in the present study were prepared in sealed colorimeter tubes and autoclaved to prevent the growth of microorganisms. Such treatment does not affect the stability, since the results obtained are in agreement with those obtained by King.

Since these solutions have only a limited stability the more reproducible standards previously reported are considered more convenient and reliable. It should be noted that the hemoglobin/hemin intensity factor to be used is determined by the nature of the filter,<sup>2</sup> and a given factor may not be carried from one filter to another.

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## A TIDAL VOLUME RECORDER FOR ANESTHETIZED DOGS

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A PRACTICAL recorder for quantitatively and continuously measuring tidal volume must fulfill several requirements. It should (1) record the tidal volume for long periods of time without demanding the attention of the investigator, (2) record accurately, during rapid and marked changes in respiratory rate, the tidal volume and duration of inspiration or expiration, and (3) leave a permanent record that easily can be converted to volume. The recorder should not require a large area for the record or present marked resistance to the flow of air.

The usual methods of recording respiration, such as lateral tracheal pressure, the chest pneumograph, or the Gaddum method,<sup>1</sup> measure the respiratory rate and, at best, relative changes in tidal volume. However, these changes are magnified or minimized by the speed of inspiration or expiration. The recorder that most nearly satisfies the requirements was that described by Wright.<sup>2</sup> Use of this recorder was limited by the space required by the apparatus.

We found that the apparatus described below fulfilled the listed requirements. Tidal volumes of anesthetized dogs have been measured for several hours without adjusting this apparatus. Respiratory rates of 2 to 60 per minute and tidal volumes of 25 to 400 cc have been recorded satisfactorily. Slow or rapid inspirations did not affect the operation of the apparatus. The maximal height of the record was about 4 cm (equivalent to 400 cc) and the base line was horizontal. The resistance of the instrument to the flow of air varied between 5 and 20 ml of water. The apparatus was mounted under the dog table.

### APPARATUS

Fig. 1 is a schematic diagram of the apparatus. Fig. 2 is a diagram of the double piston valve, drawn to scale. The numbers in the following description refer to Fig. 1 unless otherwise indicated. (1) A metal to metal contact switch operated mechanically by contact with the top of the spirometer. It is inserted into the circuit between one side of the secondary coil of the transformer and one side of the relay coil. (2) A mercury contact switch constructed as a first class lever. Electric connections are made through the mercury cup (b) and the fulcrum (f). One connection is to the transformer and the other to the relay coil. This switch activates the relay. A brief contact is sufficient because one pair of the relay contact points (d) continues the activation of the coil until

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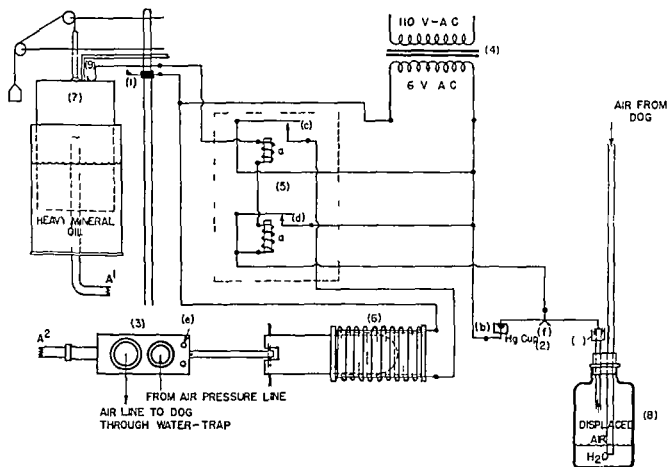
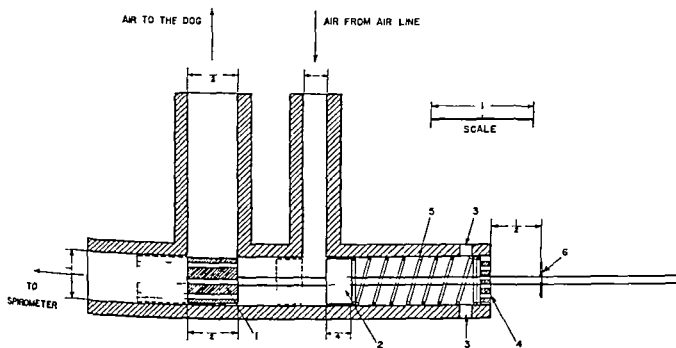


Fig 1—Schematic diagram of an automatic tidal volume recorder (1) Metal to metal contact switch (2) mercury contact switch (3) double piston valve (see Fig 2 for details) (4) 110 volt transformer (5) double contact relay (Advance Type 804B 0 volts AC) (6) solenoid (Guard Electric Co 6 volt AC 8 ounce pull) (7) spirometer of 600 c.c. capacity (8) water trap (9) spirometer stop A and B air tube connecting piston valve to spirometer (a) mercury cup valve (b) mercury contact cup (c) contact points which complete circuit to solenoid (d) contact points for activation of relay coil (e) air escape hole (f) fulcrum of mercury contact switch



DESIGNED BY W.S. WALLER  
 CO. ED. M.M. BARRATT 2 6-8

Fig 2—Double piston valve in position to direct air flow to the spirometer (The broken lines indicate the position of the valves during inspiration.) 1 Piston (perforated to permit passage of air) 2 piston (solid) which directs the flow from the air line 3 holes drilled in piston cylinder 4 perforated washer 5 spring to return the piston to the left 6 pin through piston rod to stop movement to the left at the desired position

the switch (1) is opened. Air exhaled from the dog forces one end (a) of the lever up to make the opposite end (b) contact the mercury in the cup. Water traps (8) on each side of a tracheal cannula direct the flow of air to and from the dog. A T-shaped tracheal cannula was made of 10 to 15 mm diameter glass tubing. A small side tube was added to measure tracheal pressure. All air passages were of large diameter. Movements of the spirometer were transferred to sooted kymograph paper by means of a cord connected to the spirometer and a vertically moving recording point.

The working sequence of the apparatus is as follows. When the dog is in respiratory rest the spirometer (7) is filled with air, and the switches (1), (2) and the relay (5) are open. The piston valve (3) is in position to connect the tracheal cannula with the spirometer and to direct the flow from the air line through the escape holes (e). (See Fig. 2, broken lines, for the position of the valve.)

As the dog inhales, the spirometer descends and the switch (1) closes to connect one end of the secondary coil of the transformer to the relay coil.

As the dog exhales, the switch (2) closes and completes the circuit between the opposite ends of the secondary coil and the relay coil. This activates the coil and closes the contact points. One pair of the contact points (d) continues the activation of the relay coil regardless of the position of the switch (2). The other pair of points (c) activates the solenoid (6) which pulls the double piston valve (3) to the position illustrated by the solid lines in Fig. 2. This shift in position blocks the connection between the tracheal cannula and the spirometer and directs the flow from the air line to the spirometer through the perforated piston valve. As the spirometer is filled, the switch (1) opens and breaks the connection between the transformer and the relay. This returns the apparatus to the original position ready for another respiratory cycle. Filling the spirometer requires less than a second.

#### DISCUSSION

The tidal volume recorder has been used in our laboratory as a part of routine acute experiments. The records were easily read and gave information not obtainable by simultaneous lateral tracheal pressure or by chest pneumograph methods. Epinephrine administration resulted in either a temporary decrease or an increase in tidal volume, depending on the degree of blood pressure change, followed by tidal volumes that usually were greater than the control tidal volumes. Histamine usually caused a decrease in tidal volume lasting longer than the blood pressure drop. Sodium pentobarbital and sodium barbital caused a decrease in tidal volume.

Tidal volume is influenced by simultaneous changes in pleural pressure, bronchodilatation or bronchoconstriction, duration of inspiration, and the status of the pulmonary capillary bed. Pleural pressure was shown to vary when bronchodilator or bronchoconstrictor compounds were administered.<sup>3,4,5</sup> Pleural pressure correlated with tidal volume appears to give considerable additional information even if the capillary bed status and duration of inspiration are 12

TABLE I TIDAL VOLUMES PLEURAL PRESSURES AND TIDAL VOLUME/PLEURAL PRESSURE RATIOS CALCULATED FROM FIG 3 AT THE LETTERS INDICATED

LETTERS	DRUG	TIDAL VOLUME	PLEURAL PRESSURE	TV/PP
A		160		80
B		170	5	68
C	Epinephrine 20γ	180	18	47
D		175	16	109
E		180	12	150
F	Histamine 110γ	190	20	95
G		170	40	42.5
H		180	22	82
I	Epinephrine, 20γ	180	5	72
J		190	20	95
K		190	16	119
L		190	18	106

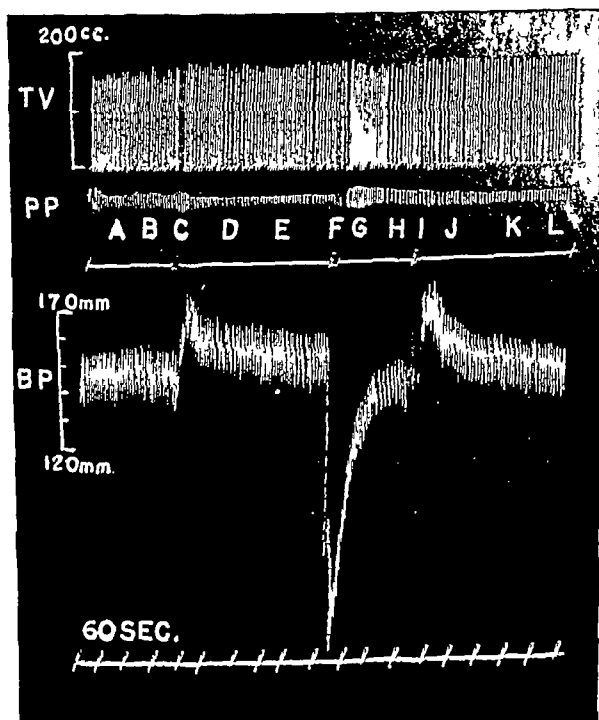


Fig 3—Effect of epinephrine and histamine on tidal volume (TV) pleural pressure (PP) and blood pressure (BP). Epinephrine 20γ injected intravenously at C and I. Histamine 110γ injected intravenously at F. See Table I for interpretation of the tracing.

noied, provided the latter changes are not too great. To obtain pleural pressure a balloon was inserted into the potential pleural cavity and attached to a small recording mercury manometer. The resulting records gave information that seems to indicate degrees of bronchodilatation or bronchoconstriction. Fig 3 and Table I demonstrate the results obtained when epinephrine and histamine were administered. Degrees of bronchodilatation and constriction have been indicated by dividing the tidal volume (TV) by the length of the line measuring the pleural pressure (PP) and are calculated at the points indicated on the tracing. A decrease in the tidal volume/pleural pressure ratio indicates bronchoconstriction, and an increase indicates bronchodilatation. Correlation of tidal volume and pleural pressure is being studied more completely in our laboratories.

#### SUMMARY

Apparatus suitable for continual, quantitative measurements of tidal volumes of anesthetized dogs is described.

A method that appears to indicate degrees of bronchodilatation or bronchoconstriction by simultaneous measurement of tidal volume and pleural pressure is presented.

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#### Erratum

In the paper by Donohue and Fremes, "Maternal Isoimmunization Without Evidence of Clinical Erythroblastosis Fetalis in the Newborn," which appeared in the May issue of the *JOURNAL* (33: 526, 1948), the column heads of Table II, p. 529, should read CDe/CDe, CDe/cDe, cDe/cDe, cDe/cDe.



# RESULTS OF VACCINATION AGAINST INFLUENZA DURING THE EPIDEMIC OF 1947

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## INTRODUCTION

THE influenza epidemics of 1943 and 1945 respectively provided the opportunity for several groups of investigators<sup>1-5</sup> to evaluate the use of combined influenza A and B virus vaccine as a means of protection against this disease. These studies yielded highly significant results in favor of the prophylactic value of vaccination even when the vaccine was given as long as a year preceding an outbreak.<sup>5</sup> Although these investigations established without doubt the importance of vaccination as a control procedure during the 1943 and 1945 epidemics, further evaluation during subsequent outbreaks in civilian groups seemed warranted. Studies on the periodicity of epidemic influenza indicated that sometime during the fall and winter of 1946-1947 an outbreak would occur. In the fall of 1946 therefore, a study was organized at the University of Chicago to test further the usefulness of vaccination for protection against influenza.

## METHODS

*Population*—Two thousand and twenty students (men and women) living in twelve University houses were employed in the epidemiologic study. Seven hundred ninety volunteers were vaccinated and 1,230 served as controls. The vaccination program was carried out in the dormitories over a period from Nov. 6 to Dec. 6, 1946. Not more than 50 per cent of the population in a given house was vaccinated. A record was kept of each student in the test and control groups in the Student Health Clinic and all respiratory illnesses were recorded. The epidemiologic data were derived from patients reporting to the clinic for care. In order to obtain crude data on the over all incidence of influenza, a questionnaire was sent to all the students in the test and control groups on April 6 inquiring whether they had had an illness with symptoms resembling influenza during the previous six weeks which included the epidemic period.

In addition to those in the dormitories several hundred students living in private homes, fraternity houses, and so forth, were also vaccinated in the

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clinic These, along with several hundred unvaccinated students not included in the epidemiologic group, were cared for in the clinic or hospital during the epidemic period Sera from both acute and convalescent phases also were obtained from many in these groups for serologic diagnosis of influenza

**Vaccines**—Three different commercially prepared inactivated virus vaccines\* having an A component of 25 per cent PR-8 and 25 per cent Weiss strains and a B component of 50 per cent Lee strain were employed The number receiving each brand of vaccine was approximately the same Each student in the test group was given 1 cc of vaccine subcutaneously in the deltoid region of the right or left arm Skin tests to determine sensitivity to the vaccines were not performed prior to vaccination The volunteers were questioned about sensitivity to eggs, fowl feathers, or to previous vaccinations Those giving positive histories were placed in the control group Only minor local reactions and an occasional moderately severe generalized reaction to the vaccines consisting of fever and malaise were encountered

### RESULTS

**Immune Response to Vaccination**—The method employed for antibody determination was Salk's<sup>7</sup> modification of the Hirst<sup>8</sup> chicken red cell agglutination inhibition technique The immune response to vaccination (Table I) was satisfactory, as shown by a comparison of the antibody titers against the virus strains in the vaccines in sera collected before and from two to three weeks after vaccination There was no significant difference in the immune response to the three vaccine preparations

TABLE I MEAN INFLUENZA ANTIBODY TITERS BEFORE AND TWO TO THREE WEEKS AFTER VACCINATION SUBCUTANEOUSLY WITH 1 CC OF COMBINED A AND B INFLUENZA VIRUS VACCINE

VACCINE PREPARATION	VIRUS STRAINS	PAIRS OF SERA	ANTIBODY TITERS	
			PREVACC	POSTVACC
Parke, Davis*	PR 8	94	374	1944
	Weiss	95	224	1015
	Lee	111	156	212
Eli Lilly†	PR 8	97	294	1070
	Weiss	94	137	914
	Lee	115	114	401
Lederle‡	PR 8	90	255	806
	Weiss	95	182	200
	Lee	93	104	400

\*Calcium phosphate adsorption inactivated by ultraviolet light

†Chicken red cell eluate inactivated by formalin

‡High-speed centrifugation inactivated by formalin

**The 1947 Epidemic**—Late in January, 1947, outbreaks of influenza, found to be due to influenza virus type A, were reported in Army population groups in California, Colorado, and New Jersey<sup>9</sup> Subsequently, the military personnel of other Army installations near Chicago were attacked During this period when the Army was having its main incidence of influenza, the civilian popula-

\*Kindly supplied by Lederle Laboratories Inc New York N Y Eli Lilly & Company Indianapolis Ind and Parke Davis & Company Detroit Mich

tion appeared to be relatively free from it. The peak of the epidemic among civilians occurred during the latter part of February and the early part of March.<sup>10 15</sup>

A sharp increase in the incidence of acute respiratory disease occurred among the student population at the University of Chicago during the first week of March, 1947. The majority presented symptoms characteristic of mild influenza with sudden onset malaise frontal headache back pain and generalized muscle pains, and nonproductive cough. Only a few complained of sore throat.

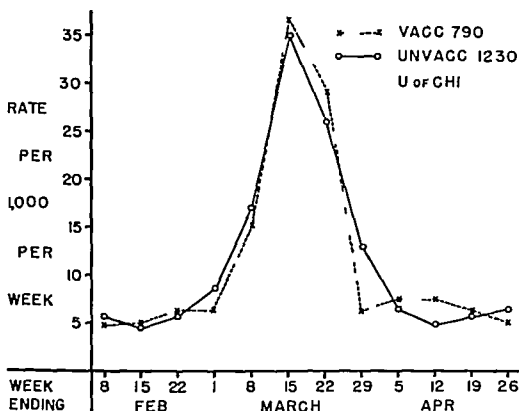


Fig 1—Incidence of acute respiratory disease among vaccinated and unvaccinated student reporting to the clinic for care during the epidemic period of influenza, 1947

or stuffy nose or suffered from nausea or vomiting. The epidemic was of short duration, reaching its peak March 15 and lasting a period of about four weeks (Fig 1). Before and after the period of increased incidence the weekly rate for respiratory disease was low and was essentially the same in the vaccinated and unvaccinated groups (Fig 1).

*Incidence*—The data shown in Table II are based on the number of students coming to the clinic and admitted to the hospital for care for acute respiratory disease. The criterion for admission to the hospital was generally a fever of  $101^{\circ}$  F or above with corresponding constitutional symptoms and cough. During the period of increased incidence of acute respiratory disease from March 2 to April 5, 1947, the attack rate was the same in the vaccinated and unvaccinated groups being 9.5 per cent. Two and five tenths per cent of the vaccinated and 2.26 per cent of the unvaccinated students were considered sufficiently ill to be admitted to the hospital (Table II). Five hundred seventy-six students from the control group and 606 from the test group returned the respiratory disease questionnaire. Forty-one and eight tenths per cent of those

answering from the control group and 46.5 per cent of the test group described symptoms of upper respiratory tract infection. The great majority dated their illness during the period of increased incidence as noted from the clinic records.

TABLE II INCIDENCE OF ACUTE RESPIRATORY DISEASE DURING EPIDEMIC PERIOD FROM MARCH 2 TO APRIL 5, 1947

GROUP TREATED IN	VACCINATED (790)		UNVACCINATED (1210)	
	NUMBER OF CASES	INCIDENCE	NUMBER OF CASES	INCIDENCE
Clinic	55	7.00	88	7.14
Hospital	20	2.50	29	2.36
Total	75	9.50	117	9.50

*Serologic Diagnosis of Influenza A*—From Feb. 8 to April 26, 1947, acute and convalescent phase blood was collected from 192 unvaccinated individuals showing respiratory illnesses suggestive of influenza. These were tested for antibody rise to the PR-8 and Lee strains of influenza virus. No significant rise was noted against the Lee strain in any of the specimens of convalescent sera. On the other hand, as is shown in Table III, 40 per cent of the blood specimens during the first three weeks of March showed a fourfold or greater rise in titer to the PR-8 strain of influenza A virus. Only two of the twenty-nine blood specimens collected before and after the period of increased incidence of illness, however, showed significant rises in titers to PR-8.

TABLE III ANTIBODY RESPONSE TO TYPE A (PR-8 STRAIN) INFLUENZA VIRUS IN UNVACCINATED INDIVIDUALS SHOWING SYMPTOMS OF INFLUENZA IN 1947

PERIOD OF TIME	MARCH						
	FEBRUARY 8-28	MARCH 2-8	MARCH 9-15	MARCH 16-22	MARCH 23-29	APRIL 30-5	APRIL 6-26
Per cent*	16	40	41	39	28	15	13
Convalescent sera*	1	16	16	16	9	2	1
Pairs of sera	62	400	390	410	320	134	77

\*Convalescent sera showing a fourfold or greater rise in titers.

*Etiological Diagnosis of Influenza*—Throat washings were collected from thirty-two hospitalized patients immediately after admission by having them gargle with 10 to 20 cc of nutrient broth. The throat washings were then frozen and kept in the dry ice chest until ready for study. Two strains of influenza virus, J-16 and L-32, were isolated by direct inoculation of 0.2 cc of the unfiltered, undiluted throat washings into the allantoic cavities of 10-day-old chick embryos on the third egg passage. After the first passage, 0.1 cc of a  $10^{-2}$  dilution of the allantoic fluid was employed. Penicillin and streptomycin (100 units per 1.0 cc of undiluted washings) were added one-half hour before the initial inoculation was made and occasionally before subsequent passages. The eggs were incubated for forty-eight hours at 99 to 100° F. All samples of throat washings were subjected to at least four egg passages before they were discarded.

*Antigenic Relationship Between Influenza A Virus PR-8 and the 1947 Strains*—Sixty-eight pairs of sera from unvaccinated individuals, collected be-

fore and after the infections again were tested for antibody levels against the PR 8 as well as against the recently isolated J 16 and L 32 strains (Table IV). Thirty-seven of the convalescent sera showing a fourfold or greater rise in antibody titers against PR 8 also showed significant rise in titers to strains J 16 and L 32. Likewise, as shown in Table IV, thirty-one pairs of sera showing no significant rise in titer to PR 8 also showed no rise to the 1947 strains.

TABLE IV. INFLUENZA ANTIBODY TITERS FOR THE PR 8, J 16 AND L 32 VIRUS STRAINS IN SERA FOLLOWING VACCINATION WITH COMBINED A AND B VACCINE CONTAINING PR 8 STRAIN AND INFECTION IN UNVACCINATED INDIVIDUALS

VIRUS STRAINS	NUMBER OF PAIRS OF SERA TESTED		
	POSTVACCINATION		CONVALESCENT
	60	MEAN FOLD INCREASE	††
PR 8	71	3.6	11
J 16	1	3.7	0.7
L 32	18	5.1	0.7

Shown fourfold or greater increase in titer at the time of original testing for PR 8 influenza antibodies.

† Shown threefold or less rise in titers to PR 8 influenza antibodies at time of original testing.

Sixty pairs of sera collected at the time of vaccination also were retested for antibody response against PR 8 and the 1947 viruses (Table IV). Whereas the postvaccination sera showed an average increase in antibody to PR 8 of over sevenfold, no significant rise was noted to the J 16 and L 32 strains. These data show that there was no close antigenic relationship between influenza viruses causing the 1947 outbreak and the influenza A component in the vaccine. Yet the increase in antibody titers in the convalescent sera to the J 16 and L 32 and also to the PR 8 strain establishes the first two as type A influenza viruses.

*Serologic Studies on Vaccinated Individuals*—Acute and convalescent phase sera were obtained from 46 vaccinated individuals in the test group who came to the clinic or were hospitalized for care between March 2 and April 5. Twenty-three pairs (50 per cent) showed a fourfold or greater rise to the J 16 strain of influenza virus. The majority of those showing significant rises showed less rise in antibody titer to the PR 8 strain, in all probability because of the initially high antibody level in the acute phase sera (Table V).

Pre and postvaccination blood specimens had been obtained from 25 of the vaccinated individuals from whom acute and convalescent sera were collected at the time of illness. Fourteen (56 per cent) showed significant rises in titer in the convalescent sera to the J 16 virus but no rise in titer to this strain in the postvaccination sera (Table V). On the other hand, vaccination provoked significant rises in titer to the PR 8 strain, and the antibody levels to PR 8 were only slightly lower in the acute phase sera collected at the time of illness than those present in the postvaccination samples (Table V). The low antibody titers to the closely related J 16 and L 32 influenza virus strains and the relatively high antibody levels to PR 8 in the acute sera of vaccinated individuals together with the significant rise in titers following infection establish the former strains as the etiologic agents of the 1947 influenza outbreak.

TABLE V COMPARISON OF INFLUENZA ANTIBODY TITERS FOR THE PR 8 AND J 16 STRAINS IN SERA FOLLOWING VACCINATION WITH COMBINED A AND B VACCINE CONTAINING PR 8 STRAIN AND INFECTION IN VACCINATED INDIVIDUALS

CASE	VIRUS STRAIN	VACCINATION SERA			INFECTION SERA		
		PRE	POST	FOLD RISE	ACUTE	CONVAL	FOLD RISE
C 99	PR 8	32	1024	10	512	4096	6
	J 16	32	32	0	16	128	6
764 D	PR 8	64	2048	10	512	1024	2
	J 16	32	64	2	32	256	6
161 Z	PR-8	64	1024	8	1024	1024	0
	J 16	32	32	0	32	128	4
197 Z	PR 8	64	8192	14	8192	8192	0
	J 16	16	32	2	64	512	6
125 B	PR-8	128	1024	6	512	1024	2
	J 16	16	16	0	16	128	6
299 B	PR 8	64	2048	10	2048	4096	2
	J 16	32	32	0	32	128	4
586 B	PR 8	4096	4096	0	2048	8192	4
	J 16	32	64	2	32	512	8
594 B	PR 8	128	2048	8	1024	4096	4
	J 16	16	16	0	16	128	6
266 B	PR 8	2048	4096	2	2048	4096	2
	J 16	64	64	0	64	512	6
1040 V	PR-8	128	4096	10	512	1024	2
	J 16	32	64	2	32	1024	10
1027 V	PR 8	64	2048	10	512	1024	2
	J 16	32	64	2	32	256	6
105 V	PR 8	256	512	2	1024	2048	2
	J 16	64	64	0	16	256	8
1057 V	PR 8	256	512	2	256	512	2
	J 16	64	64	0	128	512	4
187 V	PR 8	512	2048	4	4096	4096	0
	J 16	32	32	0	32	128	4

#### DISCUSSION

A year previous to the 1943 influenza A epidemic, Salk and associates<sup>1</sup> inoculated a large number of subjects in a state institution with a combined influenza A and B vaccine similar to one of the preparations employed in this study. The response to the vaccine was good and sera from vaccinated individuals even after a year showed antibody levels several times higher than the prevaccination titers. Proof of the immune state of the vaccinated group after a year compared with the control group was shown in the markedly lower incidence of infection in the former—1.9 per cent compared with 12.4 per cent in the latter. The larger study for the evaluation of influenza vaccines conducted by members and associates of the Commission on Influenza, Army Epidemiological Board, during the 1943 epidemic likewise yielded results significantly in favor of vaccination.<sup>1, 2</sup> The incidence of infection was 2.2 per cent in the test group and 7.1 per cent among the controls. In this study the inoculations were given immediately before and up to twelve weeks previous to the outbreak of the epidemic. Among the group vaccinated from eight to twelve weeks before the epidemic, however, essentially no protection was shown.<sup>10</sup>

The vaccination studies by Francis<sup>3</sup> and Hirst<sup>4</sup> and their associates during the influenza B epidemic of 1945 again yielded favorable results. The vaccinations were carried out within a month preceding the outbreak. Francis and

co workers observed an incidence of 1.15 per cent among the vaccinated and 9.91 per cent among the unvaccinated individuals while Hirst noted an incidence of 0.5 and 12.5 per cent respectively in the test and control groups. The virus strains isolated during the 1943 influenza A and 1945 influenza B epidemics were found to be closely related antigenically to those in the vaccines and the significantly lower incidence among the vaccinated individuals was considered to be due to the prophylactic effect of vaccination.

During the 1947 influenza A epidemic as shown in this and other reported studies, no such dramatic protection following vaccination was observed.<sup>9,12</sup> At the University of Michigan Francis Salk and Quilligan<sup>10</sup> reported an incidence of influenza in 10,328 vaccinated individuals of 7.2 per cent compared with 8.1 per cent in 7,615 controls. Likewise in a study at Bucknell University by Fowle and associates, the incidence was 7.05 per cent in vaccinated persons (1,250) and 7.3 per cent in the unvaccinated (794).<sup>11</sup> The findings in these studies also support the epidemiological observations made by the Army during the 1947 epidemic among its personnel.<sup>9</sup> Van Ravenswaay<sup>13</sup> reported an incidence of 20.2 per cent among 237 vaccinated cadets compared with 27.8 per cent among 284 nonimmunized boys. Sigel and associates<sup>14</sup> studied the 1947 epidemic outbreak among 521 students in a boys' school, 88 per cent of whom were vaccinated. Of the vaccinated group 5.4 per cent were considered to have become ill whereas 4.9 per cent of the smaller control group contracted influenza. The incidence of moderately ill patients in the vaccinated and unvaccinated groups was 3.6 and 3.0 per cent respectively. Weller, Cheever and Enders<sup>1</sup> also found no prophylactic effect following the intradermal inoculation of influenza A and B vaccine during the 1947 epidemic although the antibody response to vaccine by this route appeared to be adequate. The epidemiological data in this study were obtained by questionnaire. Of the 316 individuals who received the vaccine, 3.4 per cent reported symptoms of acute upper respiratory tract disease, while 2.8 per cent of 329 unvaccinated personnel had a similar illness during the epidemic period.

The only favorable study reported to date on the value of vaccination against epidemic influenza during 1947 is that of Trimble.<sup>12</sup> This was carried out among the student population at the University of Missouri. The interpretation of the results of this study is open to question because the incidence of influenza among the vaccinated and unvaccinated groups was obtained by polling 'at random various students encountered throughout the University campus and surroundings'. A detailed analysis of the 880 cases admitted to the hospital during the epidemic period would have yielded more definitive results.

In our study the questionnaire method yielded a crude incidence of acute respiratory disease among the vaccinated and nonvaccinated groups of 4.65 and 4.15 per cent respectively whereas the incidence during the epidemic period was the same and was only 0.95 per cent in the test and control groups as shown by the clinic data. It was still lower as far as our serologic studies were concerned. Only 38.6 per cent of 148 pairs of sera collected from students showing symptoms of influenza during the epidemic period showed significant rises

(fourfold) in titer to the PR-8 diagnostic antigen. The studies of Sigel and associates<sup>14</sup> employing the complement fixation test and soluble PR-8 antigens<sup>1</sup> showed a much greater number with significant rises.

It is evident from the etiological studies of the influenza epidemic of 1947 that the failure of the combined A and B vaccines to protect was not due to the time interval between vaccination and the outbreak. Vaccination was equally ineffective as reported by others when done from two weeks to two and three months before influenza occurred. Although in our study the interval was from four to five months, antibody determinations on acute sera in vaccinated individuals showed essentially the same high titers as did the sera taken two to three weeks after vaccination. These observations support those of Francis, Salk, and Quilligan<sup>10</sup>. In all probability the failure of the vaccine in this epidemic was due, as Francis and associates have pointed out, 'to the lack of sufficient antigenic crossing between strains of virus in the vaccine and the prevalent strains responsible for the epidemic.' This conclusion was based on evidence obtained from comparative serologic studies with strains isolated during the 1947 epidemic and those incorporated in the vaccines. Our studies on the antigenic relationship between the recently isolated strains and those making up the vaccines support the observations of Francis and associates,<sup>11</sup> as well as those of Smadel,<sup>9</sup> Hirst,<sup>18</sup> and Sigel and co-workers.<sup>19</sup> The failure to demonstrate the usefulness of the vaccine in this epidemic should not be taken as a reflection on the vaccine itself or on previous studies during which positive results were obtained. The vaccination programs of Francis, Salk, Hirst, and other members of the Influenza Commission during the 1943 and 1945 epidemics establish without doubt the value of vaccination as a control procedure against the disease caused by virus strains closely related to those in the vaccine.<sup>15</sup>

The low antibody levels against the 1947 strains in the prevaccination and acute sera indicate that the population has had little previous experience with them. Whether the 1947 strains, which were seeded throughout the population during the spring months, will be responsible for subsequent outbreaks cannot be determined. Antibody response to vaccination<sup>20</sup> with combined influenza virus A and B vaccines containing, in addition to the PR-8, Weiss, and Lee strains, the FM-1 1947 strain was disappointing. While the PR-8, Weiss, and Lee strains stimulate significant antibody response in individuals with low initial titers (16 to 64) as shown by the chicken red cell agglutination inhibition technique, the FM-1 antigen produced little or no specific antibody rise. These studies point up the fact, as Van Ravenswaay concludes,<sup>13</sup> that "further study is necessary to define the application and limitations of influenza vaccine as a prophylactic agent." Sigel and co-workers offer suggestions for improving the vaccine.<sup>14</sup> Dingle,<sup>21</sup> in reviewing the problem of influenza emphasizes the many gaps in our knowledge of this disease and its control.

#### CONCLUSIONS

No prophylactic effect was observed following vaccination with combined A and B influenza virus vaccines four to five months prior to an outbreak of



influenza among the students at the University of Chicago in March 1947. The time between vaccination and the onset of the epidemic was probably not important in accounting for the lack of effect of the vaccines. Of most importance was the absence of a close antigenic relationship between the viruses in the vaccine and the strains causing the epidemic; this has been demonstrated by virus isolation and serologic studies.

Failure to demonstrate the usefulness of the vaccine during the 1947 epidemic should not be taken as a reflection on the vaccine or on previous studies during which positive results were obtained. Studies made during the 1943 and 1945 epidemics established without doubt the value of vaccination as a control procedure against the disease caused by a virus strain closely related to the strains in the vaccines. However, a comparison of data concerning the value of vaccination during this and the 1943 and 1945 epidemics shows clearly that a vaccine which affords protection during one epidemic does not insure protection against subsequent outbreaks.

The efficiency of a vaccine will depend (1) on its ability to initiate an adequate antibody response and (2) on a close antigenic relationship between the virus components in the vaccine and those initiating the epidemic. Further development of an influenza vaccine of broad antigenic coverage and study of the limitation of its usefulness as a prophylactic agent against epidemic influenza are needed.

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## TOPICAL APPLICATION OF SUBTILIN TO TUBERCULOUS LESIONS IN MAN\*

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IN RECENT years attention has been focused upon the chemotherapeutic approach to the treatment of tuberculosis. Promin and its derivatives were found to be highly effective against *Mycobacterium tuberculosis* in vitro and in experimental animals, yet clinical experience failed to reveal demonstrable effects of these drugs on tuberculous infections in human beings. On the other hand streptomycin, which was similarly effective in vitro and in animals has proved efficacious in the treatment of certain phases of human tuberculosis. Final evaluation of an antituberculosis agent must be carried out after its use in the treatment of the disease in human beings.

In the past two years there have been several reports regarding basic investigations of a new antibiotic, subtilin which has been effective against *Mycobacterium tuberculosis* in vitro and to a limited extent in animal experiments. This is a preliminary report on the topical administration of subtilin to eight patients with tuberculous lesions. It is realized that no conclusions can be drawn from a report of this nature, but it was felt that this limited clinical experience should be made available to other investigators.

The antibiotic activity of *Bacillus subtilis* has been recognized for a number of years, and in 1944 subtilin, an antibiotic derived from a strain of *B. subtilis* was announced by Jansen and Hirschmann<sup>1</sup>. Bacteriostatic and bactericidal action in vitro against *Mycobacterium tuberculosis* was reported by Salle and Jann<sup>2</sup> and confirmed by Wong, Hambly, and Anderson<sup>3</sup>. Preliminary studies reported by Salle<sup>4</sup> indicated that experimental tuberculosis in guinea pigs might be favorably altered by administration of subtilin but experimentation in vivo was retarded by certain physical properties of the drug.

The solubility of subtilin in physiologic saline solution and in human serum is approximately 0.05 per cent, although it is readily soluble in water. As shown by Wilson, Lewis, and Humphreys<sup>5</sup> subcutaneous or intramuscular injection of strong aqueous solutions into animals produces very low concentrations of the drug in the blood and subtilin precipitates at the site of injection. The saline tissue fluids apparently precipitate the antibiotic, and subsequent absorption of the precipitate is exceedingly slow.

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Part of a cooperative study with the Division of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco, under the direction of Dr. H. H. Anderson, the Department of Bacteriology, University of California, Los Angeles, under the direction of Dr. A. J. Salle, and members of the staff of the Western Regional Research Laboratory, Albany.

The drug has not been administered orally because tests *in vitro* have revealed that digestive enzymes destroy a considerable portion of subtilin activity.<sup>7</sup>

Observations on chemical and physical properties of subtilin have been reported by Dimick, Alderton, Lewis, Lightbody and Fevold, and more recently by Lewis.<sup>8</sup>

Since subtilin was relatively insoluble in physiologic saline solution, parenteral administration was considered impractical. Although it is fully realized that aerosol administration alone is by no means a satisfactory method of treatment of tuberculosis, it was felt that useful information might be gained by employing the drug topically, and the present study was undertaken.

Topical application was by the intraoral administration of nebulized solutions of subtilin to eight patients with laryngeal or endobronchial tuberculosis.

#### OBSERVATIONS IN EIGHT CASES

The patients ranged in age from 18 to 60 years and included members of both sexes. All were observed for at least six weeks prior to subtilin therapy. As shown in Table I, four of the patients had tuberculous laryngitis and one had endobronchial disease. Three other patients had moderately or far advanced pulmonary tuberculosis with cavitation, and one of these had a recent bronchogenic dissemination of disease. These latter three patients were considered to have a conspicuous bronchial factor in their pulmonary disease although lesions were not observed in the main stem bronchi at bronchoscopic examination, and subtilin therapy was directed toward presumed endobronchial lesions in the smaller bronchi.

*Method of Administration*—At the beginning of the study subtilin\* was supplied as a 0.25 per cent solution in 0.45 per cent sterile saline solution. Subsequently the strength of subtilin in the solution was increased and the saline concentration was necessarily decreased (due to the low solubility of subtilin in saline). The daily dose was divided into four to ten portions of 0.5 to 1.0 cubic centimeter. The portion was introduced into a nebulizer† attached to a standard oxygen tank and administered intraorally as described elsewhere.<sup>9</sup>

Later a 1.8 per cent aqueous solution of urea was used as the solvent in place of saline. This isotonic solution produced less tracheal irritation.

Both 4 and 6 per cent solutions of subtilin were administered to one patient.

*Dosage*—Early in the investigation patients were given 10 mg. of subtilin daily, and the dose was increased gradually in the course of two or three months to at least 90 mg. daily. For a considerable period two patients received 90 mg. daily, four patients, 120 mg. daily, and one patient, 160 mg. daily. One patient received 600 mg. daily for six weeks.

*Clinical and Laboratory Studies*—In an effort to determine the action and possible toxicity of subtilin, the following laboratory procedures were performed.

\*The subtilin used in these studies was supplied by the Western Regional Research Laboratory, Albany, Calif.

†Kindly supplied by Vaponefrin Company, Chicago, Ill.

in addition to complete physical, bronchoscopic, x ray, and fluoroscopic examinations determination of vital capacity, measurement of daily sputum output, urinalysis, complete blood cell count, determination of sedimentation rate and of the concentration of nonprotein nitrogen and sugar in the blood and protein in the serum albumin globulin ratio, icterus index, and cephalin flocculation, bromsulfalein liver function test and phenolsulfonphthalein renal function test.

Acid fast bacilli were cultured from the sputum of all patients with one exception, before therapy. Organisms were subsequently recovered from the sputum of this patient and two years prior to subtilin therapy. Laryngeal biopsy revealed tuberculosis.

Sensitivity of the tubercle bacilli to the antimicrobial action of subtilin was determined in six cases prior to treatment.

During therapy the following procedures were carried out at weekly intervals: measurement of sputum output, culture and smear of three day sputum concentrate, determination of vital capacity and sedimentation rate, complete blood cell count, and urinalysis. Chemical analyses of blood were repeated at monthly intervals.

The progress of the disease was followed during therapy by means of frequent physical, fluoroscopic, and x ray examinations.

TABLE I. SUBTILIN INHALATION THERAPY. DATA OBTAINED FROM EIGHT PATIENTS.

PATIENT	CONDITION	SUBTILIN (TOTAL GM.)	DURATION OF THERAPY (MO.)	IMPROVEMENT
1	Tuberculous laryngitis	3.8	3	Marked
2	Tuberculous laryngitis	51.4	6	Slight
3	Tuberculous laryngitis	17.5	7	Questionable
4	Tuberculous laryngitis	12.0	2½	None
5	Endobronchial tuberculosis	22.8	10	Marked at first re- tivation later
6	Presumed endobronchial tuber- culosis	6.3	4	None
7	Presumed endobronchial tuber- culosis	15.7	6½	None
8	Irresumed endobronchial tuber- culosis	7.7	4	None

#### *Clinical Observations—*

**Laryngitis.** In the four patients with tuberculous laryngitis, improvement was marked in one, slight in one, and questionable in one. In the fourth the disease progressed and the patient died.

The patient who showed marked improvement (Patient 1, Table I) had moderately advanced pulmonary tuberculosis with cavitation. Prior to subtilin therapy this patient had undergone therapeutic pneumothorax and phrenicectomy. The laryngeal lesions had been treated previously with penicillin inhalation and local application of sulfadiazine crystals in an effort to control secondary infection, and the disease process revealed slight improvement prior to application of subtilin. A large cavity had not responded to pneumothorax. The laryngitis improved rapidly after institution of subtilin therapy and after four months only scarring of the vocal cords remained. No change in the larynx occurred in six months thereafter, although reactivation of pulmonary disease

occurred. Since marked improvement in the laryngeal lesions occurred within one week of the beginning of subtilin therapy and while the dose was only 10 mg daily, the relationship of the antimicrobial therapy to the disease regression is difficult to evaluate.

The patient whose condition showed slight improvement (Patient 2) had active pulmonary disease. After two and a half months of subtilin therapy there was moderate subjective and slight objective improvement. Despite continuation of subtilin therapy for a total of six months, no further improvement occurred.

The patient showing questionable improvement (Patient 3) had far advanced pulmonary tuberculosis confined to the right lung. Subtilin inhalations were originally instituted to observe the possible effect on the pulmonary lesions. Despite the development and subsidence of a right pleural effusion during subtilin therapy, the parenchymal disease showed slight gradual healing in x-ray studies. Hoarseness also developed while this patient was being treated, and laryngoscopic examination revealed lesions consistent with tuberculous laryngitis. Symptoms remained mild while use of the drug was continued, but after discontinuance the laryngeal disease showed progression, both subjectively and objectively. Whether this demonstrates any effect of subtilin on the laryngeal lesions is very doubtful.

Patient 4 had advanced pulmonary tuberculosis complicated by very severe and deeply infiltrated tuberculous laryngitis and, as later shown at autopsy, obstructive involvement of the esophagus. The laryngitis progressed during subtilin therapy. The possibility of influencing a process of this nature by topical application of a drug to the larynx is unlikely.

*Endobronchial Disease.* Therapy in one subject (Patient 5) could be carefully evaluated because of extensive lesions visible at bronchoscopy. Before treatment the left main stem bronchus of this patient was 80 per cent occluded by tuberculous granulations and debris. After three and a half months of subtilin therapy, during which the dose was gradually increased to 30 mg daily, bronchoscopy revealed definite evidence of healing and only 20 per cent occlusion. After eight and a half months of therapy, during which the dose was increased to 120 mg daily, bronchoscopy revealed no occlusion or other evidence of endobronchial disease. During subtilin therapy there was no appreciable change in the far advanced bilateral pulmonary tuberculosis, no increase in vital capacity (900 cc), and no conversion of the positive results of examination of the sputum. After ten months of subtilin therapy, another bronchoscopic examination revealed inflammation and granulations involving pharynx, larynx, trachea, and bronchi. This was interpreted as being tuberculous and recent in origin. Sputum cultures at this time revealed *Myco tuberculosis* organisms which were twice as resistant to subtilin as organisms recovered in this case before treatment. It seems possible that subtilin favorably influenced the endobronchial disease originally, but it had no demonstrable effect on the pulmonary parenchymal disease. Although the development of marked bacterial resistance to subtilin was suspected, this was not proved by cultural studies, and the cause of the reactivation of endobronchial disease is unknown.

The condition in the three patients in whom endobronchial tuberculosis was presumed to be present in the small bronchi (Patients 6, 7 and 8) showed no significant change during subtilin therapy. In the absence of visible endobronchial lesions, evaluation of therapy was based on clinical condition and roentgenographic change in parenchymal disease. While there was no radiologic evidence of improvement in the extensive pulmonary disease, progression of parenchymal lesions was not demonstrated. In the patient in whom bronchogenic dissemination had occurred (Patient 8) the disease had been progressive at the beginning of administration of subtilin.

*Laboratory Studies*—Strains of *Mycobacterium tuberculosis* isolated from six of the eight patients prior to therapy showed no growth in subtilin dilutions of 1:400,000 (Patients 5, 6 and 8), 1:200,000 (Patients 3 and 7) and 1:100,000 (Patient 1)\*. After three and a half months of therapy the organisms isolated from three of these six patients (Patients 6, 7 and 8) showed no growth in solutions of subtilin four times the original effective concentrations. This decrease in bacterial sensitivity is probably beyond the experimental error of the procedure and is considered suggestive evidence of the development of bacterial resistance to subtilin *in vivo*. In one subject (Patient 5) only a twofold decrease in sensitivity was demonstrable after eight and a half months of therapy. This change may be within the experimental error of the test.

Determination of the concentration of subtilin in the blood was carried out in a patient who had received the drug by inhalation for three months in the last two weeks of which the dose had been 400 mg daily. No subtilin activity could be demonstrated in the blood.<sup>10</sup>

*Toxicity*—No evidence of toxicity in the kidneys, liver or bone marrow was encountered.

Administration of subtilin by inhalation was associated with irritation of the respiratory tree in almost all patients. Mild dyspnea, increase in cough and sputum, and mild pharyngitis occurred commonly during the first two or three weeks, after which the symptoms subsided or disappeared. The dyspnea and cough were frequently relieved by including from 2 to 4 drops of 0.25 per cent aqueous solution of Neosynephrin hydrochloride in the nebulized solution. Frank wheezing and sibilant rales were not associated with this reaction. In one case recurrent hemoptysis was encountered and in several others isolated episodes occurred.

Recurrent headaches developed in one patient receiving subtilin and there were several isolated instances of headache among the patients receiving the drug. No neurologic abnormalities were found and when headache was encountered it responded to the usual medications. Implication of subtilin as a causative agent does not seem warranted.

Asthma developed in one subject (Patient 1) after three months of therapy and it was necessary to discontinue administration of the drug for this reason. No other evidence of hypersensitivity was observed.

It should be emphasized that the lack of serious toxicity encountered in the clinical use of this drug may be inherent in the mode of administration which results in negligible systemic absorption of subtilin.

## COMMENT

It is fully realized that a drug should be administered systemically in order to exert a significant bacteriostatic or bactericidal effect in a disease such as tuberculosis in man. It is also fully appreciated that a large number of patients should be treated before any definite statement can be made regarding the clinical value of an antituberculosis agent. This preliminary investigation of the topical application of subtilin in a small number of cases does not warrant drawing any conclusions. However, suggestive evidence of therapeutic activity and of minimal toxicity affords stimulus for continued research.

Basic investigation is being directed toward the development of a modification of subtilin that can be administered parenterally. Intravenous infusion of very dilute solutions is possible in animals,<sup>11</sup> but utilization of this technique in man for a prolonged period would be impractical. The development of derivatives of subtilin with increased solubility in physiologic fluids and with retention of potency is being investigated by Lewis and associates. Preliminary results with some of these derivatives are encouraging. Work is also being carried on by Maclay and others regarding the possibility of combining subtilin with pectin to alter its characteristics of absorption.

Further investigation of the therapeutic possibilities of subtilin must await basic research directed toward development of derivatives more soluble in physiologic fluids. Subtilin in its present form has no place in the treatment of tuberculosis other than for purely investigative purposes.

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# ADMINISTRATION OF PENICILLIN AND STREPTOMYCIN BY MEANS OF THE HYPOSPRAY APPARATUS (JET INJECTION) ABSORPTION, TOXICITY AND STABILITY

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RECENTLY a device for the parenteral administration of solutions or suspensions of drugs known as the Microjet or Hypospray became available for study. Preliminary reports on its use clinically have been published by Hingson and Hughes<sup>1</sup> and by Hingson and associates. The principle of the instrument is based upon the fact that an extremely fine high pressure jet is capable of piercing the human skin with at most only slight pain. The instrument injects the drug in solution or suspension by means of high pressure\* obtained through the release by means of a button of a previously wound calibrated high tension spring which is attached to a plunger. The material to be injected is placed in a metal ampule (Metapule). It has a capacity of 0.25 cc and is shaped like a blunt nosed bullet with an orifice 0.003 inch in diameter in the rounded tip while the butt end is stoppered with a rubber plug. The tip of the Metapule is held against the skin at the site of the injection with the base locked securely in the apparatus. The plunger explodes against this rubber stopper which forces the material out of the Metapule and through the skin as a fine spray. The material is deposited subcutaneously and intramuscularly to depths varying from 0.2 to 2 cm depending on the tension of the spring and the site of injection.<sup>1</sup> Since the orifice in the Metapule through which the medication is expelled is only 0.003 inch the size of a human hair pain is either nonexistent or very slight because there is little trauma to the tissues and a minimum of pain nerve fibers is stimulated. These factors are discussed in detail elsewhere.<sup>1</sup> Another advantage attributed to this instrument is that it eliminates the fear incident to injection by needle and syringe.

## ABSORPTION

Since many infections amenable to penicillin and streptomycin therapy require injections at regular intervals throughout the day, the availability of this instrument for the administration of these antibiotics would considerably facilitate therapy. A study of the absorption of penicillin and streptomycin following intramuscular injection was undertaken. Doses of 50,000, 100,000 and 200,000 units of penicillin and of 0.05 and 0.1 Gm of streptomycin were employed. The latter dose of both antibiotics was found to be the maximum amount of each drug that could be dissolved in 0.25 cc of solution (the capacity of a Metapule).

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The theoretical pressure in the Metapule is about 4 kg per square centimeter. This is proportional however to the much smaller pressure of 11 Gm exerted on the fine jet or column of liquid in the orifice of the Metapule. The jet or column exerts a pressure varying from 300 to 3,500 pounds per square inch on the skin through which it makes a minute hole or channel.

The percentage of patients having assayable concentrations of penicillin in the blood following the injection of 50,000, 100,000, and 200,000 units of penicillin by means of the Hypospray is shown in Table I. Following the injection of 50,000 units, detectable concentrations in the blood were found in all of the patients at one hour and in 94 per cent at two hours. Measurable blood concentrations were found in only 63 per cent of the patients at the third hour and

TABLE I PERCENTAGE OF PATIENTS WITH MEASURABLE CONCENTRATIONS IN THE BLOOD FOLLOWING THE INJECTION OF VARIOUS DOSES OF BUFFERED CRYSTALLINE SODIUM PENICILLIN G BY MEANS OF THE HYPOSPRAY APPARATUS

DOSE (UNITS)	PERCENTAGE OF PATIENTS					
	HOUR					
	1	2	3	4	6	8
50,000	100	94	63	31		
100,000		100		100	50	0
200,000				100	86	14

in 31 per cent at the fourth hour after injection. When 100,000 units were administered, assayable concentrations were detected in the blood of all patients at four hours, in 50 per cent at six hours, and in none of the patients at eight hours after administration. Of the patients who were given 200,000 units of penicillin by this method, measurable concentrations were demonstrated in the blood of all the patients at four hours and in 86 per cent six hours after injection. After eight hours only 14 per cent of the patients were found to have demonstrable penicillin concentrations in the blood.

In order to compare the height and duration of the concentration of penicillin in the blood following administration by the Hypospray with that by the

TABLE II CONCENTRATIONS OF PENICILLIN IN THE BLOOD FOLLOWING THE INTRAMUSCULAR INJECTION OF 50,000 UNITS OF BUFFERED CRYSTALLINE SODIUM PENICILLIN G WITH THE HYPOSPRAY APPARATUS (0.25 cc) AND BY THE NEEDLE AND SYRINGE (1.5 cc)

PATIENT	HOUR		
	2	3	4
<i>Hypospray apparatus</i>			
1	12	06	06
2	12	06	0
3	25	06	06
4	06	0	0
5	--	06	--
6	--	06	--
7	--	12	06
8	25	06	0
9	06	0	06
10	5	06	57
Per cent	100	80	
<i>Needle and syringe</i>			
1	12	06	0
2	06	0	06
3	06	0	0
4	06	0	0
5	12	0	--
6	12	0	0
7	12	03	0
8	06	0	12
Per cent	100	25	

needle and syringe 50,000 units were given to a group of patients by both methods, and blood for penicillin assay was taken two three and four hours thereafter. The volume of the penicillin solution was 15 cc when given by needle and syringe and 0.25 cc when the Hypospray was employed. It can be seen from Table II that the concentrations obtained with the Hypospray were somewhat higher and more prolonged than those obtained with the use of the needle and syringe. Although the volumes of the solution injection were not the same, it is believed that that does not account for the differences. It is our opinion that the differences may be due to the fact that part of the penicillin injected with the Hypospray is deposited subcutaneously. It has been demonstrated that the concentrations of penicillin in the blood are prolonged following subcutaneous injection as compared with the other methods of parenteral injection.

TABLE III. AVERAGE CONCENTRATIONS OF STREPTOMYCIN IN THE BLOOD FOLLOWING THE INJECTION OF VARIOUS DOSES OF STREPTOMYCIN BY MEANS OF THE HYPOSPRAY APPARATUS

DOSE (GM)	AVERAGE CONCENTRATION IN THE BLOOD ( $\mu\text{G}/\text{C.C.}$ )		
	HOUR		
	4	8	12
0.05	1.25	0.62	0.31
0.1	2.5	1.25	0.62

The mean concentrations of streptomycin in the blood at four eight and twelve hours following the injection of 0.05 and 0.1 Gm of streptomycin are shown in Table III. At four, eight and twelve hours after the administration of 0.05 Gm of streptomycin the mean concentrations were 1.25, 0.62 and 0.31  $\mu\text{g}$  per cubic centimeter of serum respectively. Following the injection of 0.1 Gm the mean concentrations were 2.5, 1.25 and 0.625  $\mu\text{g}$  per cubic centimeter of serum at the same times.

TABLE IV. CONCENTRATIONS OF STREPTOMYCIN IN THE BLOOD FOLLOWING THE INJECTION OF 0.1 GM STREPTOMYCIN EVERY TWELVE HOURS BY THE HYPOSPRAY APPARATUS AND WITH NEEDLE AND SYRINGE

PATIENT	CONCENTRATION IN THE BLOOD ( $\mu\text{G}/\text{C.C.}$ )							
	HOUR							
	12	24	36	48	60	72	84	96
<i>Needle and syringe</i>								
1	6	6	6	6	6	6	1.25	2.5
2	6	6	1.25	6	6	1.25	1.25	1.25
3	3	6	6	6	6	6	6	6
4	6	6	6	6	6	6	6	6
<i>Hypospray apparatus</i>								
1	3	6	1.25	6	6	6	6	6
2	6	1.25	6	6	1.25	1.25	6	1.25
3	3	6	1.25	6	6	3	6	3
4	2.5	2.5	2.5	0	1.25	1.25	5.0	2.5

Since significant concentrations were obtained twelve hours following the injection of 0.1 Gm of streptomycin it was decided to investigate the results of repeated injections of this dose at twelve hour intervals and also to compare these results with the same dose given by needle and syringe. For this study,

blood for streptomycin assay was taken every twelve hours, immediately before the next injection (twelve hours following the preceding injection), over a period of four days. It can be seen from Table IV that there was no added effect from repeated injections and that the concentrations in the blood at the twelfth hour were at a plateau. Furthermore there was essentially no difference in the results obtained with the two methods of administration. Several patients were given 0.1 Gm. of streptomycin every four hours five times a day by means of the Hypospray. Assay of the blood at the fourth hour (immediately before the next injection) revealed constant concentrations at that hour, indicating no increment of streptomycin in the blood as a result of repeated injections.

The instrument employed in these studies had a spring which when released exerted a static pressure of 2,300 pounds per square inch on the skin. Although it has been shown that the depth and spread of the injected material varies with the site of injection and the race of the patient,<sup>1</sup> no differences in the blood concentrations of these antibiotics were noted in our series despite the fact that the sites of injection were varied (flexor and extensor aspects of arms, thighs, and buttocks) and both white and Negro patients were studied.

#### TOXICITY

Most patients complained of momentary stinging at the site of injection particularly with the larger doses of penicillin and streptomycin. Of the sixty patients injected in this series, only four have shown an unfavorable reaction. One patient developed a small area of induration at the site of injection after twenty-four hours. This abated after twenty-four hours following treatment with warm, wet dressings. Two patients developed small hematomata at the sites of injection. A fourth patient developed a small, subcutaneous nodule at the site of injection several days after administration. This persisted for a period of two weeks. Similar reactions have been reported by others.<sup>2</sup> Although it is relatively simple to master the technique of administration with the Hypospray, small linear cuts, blister formation, and bleeding were obtained as the result of improper handling during the early use of this instrument. The area prepared for injection should be allowed to dry before administration is attempted in order to prevent the instrument from slipping on the skin.

One patient with tuberculosis was treated with five injections per day of 0.1 Gm. streptomycin every four hours. The posterior aspects of the arms and thighs were rotated as the site of injection. After about ten days of therapy, small nodules developed at the site of injection within twenty-four hours after each injection. It is believed that these reactions were the result of repeated subcutaneous depositions of part of the streptomycin during administration with the Hypospray apparatus. In support of this are the results of absorption studies, reported herein, which indicate that some of the drug was deposited subcutaneously.

There is also evidence to indicate that the high concentrations or both streptomycin and penicillin may have been a factor in the development of the few untoward local reactions observed with the use of the Hypospray in this series.

These studies were for the most part carried out on patients with tuberculosis. After the first few patients had received injections by this instrument there was no problem in securing volunteers for the study in spite of the fact that several blood specimens were to be taken from each patient. This indeed testifies to the efficiency of this device particularly when the attitude of these patients is considered.

#### STABILITY

Our data on the stability of several aqueous solutions of penicillin stored in Metapules and employed in these studies are shown in Table V. The instability of penicillin in aqueous solution is well established. These results indicate that the stability of aqueous solutions of penicillin stored in Metapules remains undependable. The apparent discrepancy in some of the results shown in Table V is undoubtedly explained by the facts that the hand method of filling the Metapules and the method of assay are subject to error.

TABLE V. STABILITY OF SOLUTIONS OF BUFFERED CRYSTALLINE SODIUM PENICILLIN C DISPENSED IN METAPULES (0.25 CC CAPACITY) EMPLOYED IN ADMINISTERING DRUGS BY MEANS OF THE HYPOSPRAY APPARATUS

LOT	DOSE (UNITS)	BASE ASSAY (UNITS)	WEIGHTS AFTER BASE ASSAY†		
			1	5	10
			UNITS		
1	50 000	44 000	44 500	7 500	36 000
2	50 000	34 500		30 250	25 000
3	50 000	45 000		53 000	
4	100 000	94 000	84 000	115 000	50 000

\*Two to three days after preparation not refrigerated during interval

†Refrigerated

#### COMMENT

Hingson and associates have demonstrated that gonorrhea can be treated as effectively with this apparatus as with needle and syringe. Dispensing penicillin in solution in Metapules at present for general commercial use with the present design of the apparatus is at least very impractical. With the available instrument the dose of the antibiotics is limited by the size of the Metapule. This method of administration is not feasible particularly in the treatment of infections requiring large doses of streptomycin. It appears, however, that the problem of dispensing penicillin and streptomycin will be solved.<sup>3</sup> The instrument has been improved so that it will discharge the contents of 0.5 and 1 cc Metapules.

The possibility of drugs being deposited in blood vessels during the course of injection with the Hypospray was anticipated. In spite of deliberate efforts to deposit some of the drugs injected directly into blood vessels this has been accomplished only once when injection was made directly over a vein.<sup>3</sup> With most drugs this would be relatively unimportant but there are some drugs which would have serious consequences following injection directly into the circulation. Further investigation is necessary to resolve this problem completely.

## SUMMARY

These studies are evidence that penicillin can be administered by means of the Hypospray apparatus. Furthermore the device has the advantage of decreasing the pain and fear of injection which attend available methods. There are several problems to be solved before the device can be used generally in clinical practice.

We wish to thank Dr. William A. Randall and Dr. Clifford V. Price and Miss Velma L. Chandler for their assistance in the conduct of these studies.

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# THE USE OF YEAST PHASE ANTIGENS IN A COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS

## I. PRELIMINARY RESULTS WITH RABBIT SERA

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THE coincidence of pulmonary calcifications and skin sensitivity to histoplasmin<sup>1</sup> has suggested either a higher rate of infection with *Histoplasma capsulatum* than previously recognized or infection with an immunologically cross reacting antigen. A recent report<sup>2</sup> that *H. capsulatum* had been isolated from nonfatal cases implies that infection with this agent may not be uniformly fatal. The high prevalence of skin sensitive reactors to histoplasmin in the eastern central states warrants the search for additional tools for evaluating evidence of past or present infections with *H. capsulatum*. Immunologic procedures properly developed and controlled may offer additional information.

Skin test antigens (histoplasmin) are prepared from broth filtrates of the mycelial phase of *H. capsulatum*. Since it is the yeast phase of the organism that appears in affected tissues of man and animal studies utilizing this phase of the infectious agent may yield additional and possibly more specific results. The ready growth and maintenance of *H. capsulatum* in the yeast phase has been reported previously by one of us (C.C.C.) and by others<sup>3, 4</sup>. The utilization of this antigen in preliminary complement fixation studies in experimental animals is herein described. Both viable and nonviable antigens were used in immunization. Since no appreciable differences were noted other than a slightly greater immunogenic capacity of the viable forms the experiments described below are those utilizing killed antigens.

## MATERIALS AND METHODS

**Antigens.**—Three strains of *H. capsulatum* in the yeast phase which we call G 2, G 5 and G 6 were used†. Two strains of *Blastomyces dermatitidis* (A 1 and A 5), one strain of *Blastomyces brasiliensis* (B 2) and AMIS E 11 strain of *Candida albicans* were also studied‡. All of the cultures were grown in the yeast phase on glucose cystine agar slants at 37° C for five to fifteen days. The growth was washed off with sterile buffered saline and the pooled washings were filtered through sterile gauze placed in a sterile rubber stoppered vial and heated at 56° C for four hours. This constituted the stock antigen. For each series of tests calculated amounts of this stock suspension were withdrawn, washed

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†The G 2, G 5 and G 6 strains of *H. capsulatum* were reverted from the mycelial phase of strains 71, 90, and 640 respectively from the collection of Dr. N. F. Conant at Duke University.

‡A 1 strain of *B. dermatitidis* and A (Duke 930) and B strain of *B. brasiliensis* (Duke 871B) are all isolates from human subjects received from Dr. Conant at Duke University. The E 11 strain of *C. albicans* was isolated from a patient at the Army Medical Center.

two times with buffered saline, and diluted for use. The optimal antigen dilution was then determined by titration as that amount which gave the highest titer with homologous antigen and was not hemolytic or anticomplementary. The stock antigen retains its titer for several weeks when stored in the refrigerator. The portions removed for the daily test were heated at 56° C for thirty minutes to eliminate any possible anticomplementary activity.

**Preparation of Sera**—Young, healthy, albino rabbits weighing 5 to 7 pound were immunized with yeast phase antigens of *H. capsulatum* in the following manner. The organisms grown on cystine agar slants for five days at 37° C were washed off with 0.5 per cent formalized isotonic saline and stored overnight at 3 to 4° C. The suspensions were then washed three times with physiologic saline and resuspended in 0.1 per cent formalized saline to a turbidity equal to the No. 3 MacFarland nephelometer standard. Rabbits were injected intravenously for six successive days with the following amounts: 0.1, 0.2, 0.5, 1.0, 2.0, and 2.0 milliliters. Booster injections of 2 ml were given one and two weeks after the last injection and the rabbits were bled six days after the last booster. The same procedure was used for the production of sera against the antigens of the other organisms used in this study.

**Complement Fixation Test**—The complement fixation test of Kent and Rein,<sup>10</sup> developed at the Army Medical School, was employed in these studies.

**Diluent** For all dilutions 0.85 per cent salt solution buffered to pH 7.3 with 0.005M phosphate was employed.

**Sheep's Red Blood Cells** A suspension of 2 per cent washed packed cells was prepared from blood collected aseptically into an equal volume of modified Alsever's solution.<sup>11</sup>

**Hemolysin** Hemolysin prepared by the immunization of rabbits was used in an optimal dilution beyond which further decrease in dilution failed to diminish the quantity of complement required for fifty per cent hemolysis.<sup>12</sup> An equal volume of this optimally diluted hemolysin was poured into the cell suspension and mixed by ten successive pourings and at least ten minutes at room temperature were allowed for sensitization.

**Complement** Lyophilized commercial guinea pig serum, 7.0 ml, was rehydrated with 5.0 ml of the accompanying preservative containing 2 per cent boric acid and 3 per cent sodium acetate. A stock 1:20 dilution was prepared by adding 19 parts of salt solution to 1 part of rehydrated serum. The complement was titrated by the method of Kent, Bukantz, and Rein<sup>13</sup> for the determination of the 50 per cent unit of complement, and 3 units were employed in the test.

TABLE I. COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS

	TEST		REAGENT CONTROLS		
	WITH ANTIGEN	SERUM CONTROL	ANTIGEN	COMPLEMENT	SENSITIZED CELLS
Serum dilutions (ml)	0.2	0.2			
Complement (ml) (3 units)	0.2	0.2	0.2	0.2	
Antigen (ml)	0.2		0.2		0.6
Salt solution (ml)		0.2	0.2	0.4	
<i>Fixation overnight at 36° C</i>					
Sensitized cells (ml)	0.4	0.4	0.4	0.4	0.4
<i>Incubation for thirty minutes at 37° C</i>					

Degrees of reaction in tests are read according to the percentage of cells remaining unhemolyzed. The tube with the greatest dilution of serum showing 50 per cent or less of hemolysis represents the titer of the serum. It is recommended that color standards representing 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 per cent hemolysis be employed to assist in determining the aforementioned grades of reaction.

**Test** The test was conducted with suitable controls as indicated in Table I. The reagents were added in the order given and the tubes thoroughly mixed. The tubes were then placed in the refrigerator at 3 to 6° C overnight. Sheep's cells sensitized at least ten minutes previously were then added, the tubes were shaken and incubated in a 37° C water bath for thirty minutes. The tubes were then centrifuged at 7,000 revolutions per minute.



for five minutes and the tests were read against a diffuse source of light in terms of percent hemolysis using control standard tubes for comparison. The greatest serum dilutions showing 50 per cent or less of hemolysis represent the titer of the complement fixing antibodies.

### RESULTS

**Experiment 1, Titration of Antigen**—Each lot of antigen was titrated to determine the optimal dilution. The antigenic unit was considered to be the greatest concentration contained in 0.2 ml. beyond which further increase failed to enhance appreciably the serum reaction. It was required that the optimal concentration be neither hemolytic nor anticomplementary. It was found that the optimal dilution usually lay between 1:20 and 1:50 of the stock solution depending on the concentration of the latter. Table II demonstrates the determination of the optimal antigen dilution of the G 2 antigen. The tubes containing the 1:40 dilution of antigen showed no traces of anticomplementary activity and likewise reacted with the antiserum to give clear cut fixation of complement. The next dilution of antigen (1:53.3) reacted even more favorably. Thus an intermediate dilution of 1:50 was selected arbitrarily as the optimal amount and was used in subsequent studies.

TABLE II DETERMINATION OF THE OPTIMAL DILUTION OF ANTIGEN USING THE G 2 STRAIN OF *H. CAPSULATUM* AND SERUM PREPARED IN RABBITS AGAINST G 2

DILUTION OF ANTIGEN (0.2 ML.)	POSITIVE SERUM DILUTIONS IN PER CENT (0.2 ml.)					CONTROLS (%)		
	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CELLS
1:10	0	0	0	10	95	97	100	0
1:13.3	0	0	0	25	95	97		
1:20	0	0	0	25	95	97		
1:26.6	0	0	0	30	95	97		
1:40*	0	0	0	35	95	100		
1:53.3*	0	0	0	20	60	100		
1:80	0	0	0	30	90	100		
Serum control	100	100	100	100	100			

0 No hemolysis 100 complete hemolysis

\*Optimal dilution lies between 1:40 and 1:53.3 since there is good fixation but no trace of anticomplementary activity. For tests 1:50 was employed.

**Experiment 2**—Three sets of rabbit antisera prepared against three strains of *H. capsulatum* (G 2, G 5, G 6) were titrated against homologous and heterologous strains of these organisms as well as against yeast phase antigens of two strains of *B. dermatitidis* and one of *B. brasiliensis*. Fixation of complement was obtained in relatively similar titers with all combinations of histoplasma yeast phase antigens and histoplasma antisera. For example as noted in Table III sera prepared against G 2 histoplasma antigen fixed complement in the presence of its specific antigen to a titer of 1:160. In the presence of the G 2 antigen G 5 and G 6 antisera bound complement in dilutions of 1:80. Sera prepared against *C. albicans* yeast phase *Sporotrichum schenckii*\*<sup>14</sup> and *Cryptococcus neoformans*\* gave negative complement fixation results in the presence of the histoplasma G 2 antigen, while *B. dermatitidis* serum fixed complement only in dilutions up to 1:10.

Isolated from patient material at the Army Medical Center

TABLE III REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF THE G 2 STRAIN OF *H. CAPSULATUM* AS ANTIGEN AND THE SERA OF RABBITS INOCULATED WITH THE G 2, G 5, AND G 6 STRAINS OF *H. CAPSULATUM*, THE A 1 AND A 5 STRAINS OF *B. DERMATITIDIS*, *B. BRASILIENSIS*, *S. SCHENCKII*, *C. NEOFORMANS* AND *C. ALBICANS*, AND NORMAL CONTROL RABBIT SERUM

SERA	SERUM DILUTIONS (%)					CONTROLS (%)		
	1 10	1 20	1 40	1 80	1 160	ANTIGEN	COMPLEMENT	CELLS
G 2	0	0	0	0	20	100	100	0
G 5	0	10	40	50	95	100		
G 6	0	0	10	40	95	100		
A 1	10	100	100	100	100	100		
A 5	10	100	100	100	100	100		
<i>B. brasiliensis</i>	95	100	100	100	100	100		
<i>Sporotrichum</i>	100	100	100	100	100	100		
<i>Cryptococcus</i>	90	100	100	100	100	100		
<i>Candida</i>	100	100	100	100	100	100		
Normal	100	100	100	100	100	100		

0 No hemolysis (complete fixation) 100 complete hemolysis (no fixation)

The titer was taken as being the highest dilution of serum showing 50 per cent or less of hemolysis

When the G-5 antigen was used (Table IV), G-2, G 5, and G 6 antisera fixed complement in dilutions of 1 160, 1 80, and 1 80 respectively. Again no fixation was noted with sera prepared against the other fungi studied with the exception of *B. dermatitidis* serum which fixed complement in low titer. Similar results were obtained using G-6 antigen and homologous and heterologous sera.

TABLE IV REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF THE G 5 STRAIN OF *H. CAPSULATUM* AS ANTIGEN AND THE SAME SERA USED IN TABLE III

SERA	SERUM DILUTIONS (%)					CONTROLS (%)		
	1 10	1 20	1 40	1 80	1 160	ANTIGEN	COMPLEMENT	CELLS
G 2	0	0	0	0	20	100	100	0
G 5	20	0	10	40	65	100		
G 6	0	0	0	15	70	100		
A 1	10	100	100	100	100	100		
A 5	10	95	100	100	100	100		
<i>B. brasiliensis</i>	95	100	100	100	100	100		
<i>Sporotrichum</i>	100	100	100	100	100	100		
<i>Cryptococcus</i>	90	100	100	100	100	100		
<i>Candida</i>	100	100	100	100	100	100		
Normal	100	100	100	100	100	100		

See footnotes to Table III

*Experiment 3*—Strains of blastomyces and histoplasma have been found to cross react in the skin testing of animals infected with these agents<sup>10</sup> as well as in complement fixation tests employing mycelial phase histoplasma and blastomycin as antigens.<sup>6-10</sup> In Experiment 2 it was noted that blastomycin antiserum in low dilution fixed complement in the presence of yeast phase antigens of *H. capsulatum*. In order more fully to evaluate this possible relationship, complement fixation tests were done employing yeast phase blastomycin antigens in the presence of blastomyces and histoplasma antisera.

In our hands the immunogenic capacity of *B. dermatitidis* in rabbits has been far inferior to that of *H. capsulatum*. Despite repeated booster injections the highest titer of complement fixing antibodies was 1:40. When the A 5 antigen of *B. dermatitidis* was employed (Table V) in contrast to the 1:40 titers of the blastomyces antisera the G 2 (5 and G 6 histoplasma antisera fixed complement at dilutions of 1:80, 1:80 and 1:20 respectively. At first glance it would appear that the results obtained using blastomyces antigen show cross reactions too great to allow proper evaluation. However when the titers of the histoplasma antisera against the blastomyces antigen are evaluated in terms of their specific titer (compare Tables III and IV with Table V) it will be seen that the nonspecific titers are usually one fourth to one half of the specific titers, whereas the 1:40 titer obtained with blastomyces serum against its specific antigen actually represents maximum titer. Nevertheless the specificity of the

TABLE V. CROSS REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING THE A 5 STRAIN OF *B. DERMATITIDIS* AS ANTIGEN AND SERA OF RABBITS INOCULATED WITH THE A 1 AND A 5 BLASTOMYCES ANTIGENS AND THE G 2, G 5 AND G 6 HISTOPLASMA ANTIGENS

SERA	SIFUM DILUTIONS (%)					CONTROLS (%)		
	1:10	1:20	1:40	1:80	1:160	ANTIGEN	COMPLEMENT	CHITS
A 1	0	10	25	100	100	100	100	0
A 5	0	0	10	100	100	100		
G 2	0	0	0	30	60	100		
G 5	0	0	0	15	95	100		
G 6	0	50	80	100	100	100		

See footnotes to Table III

complement fixation test using histoplasma antigen in Experiment 2 was greater than that in which blastomyces antigen was used. This type of cross reaction suggests the presence of a common antigenic component shared by *H. capsulatum* and *B. dermatitidis* in which diluted histoplasma antiserum is capable of reacting with the histoplasma antigen carried by both organisms but in which blastomyces antiserum will not react as readily with histoplasma antigen. This may be due to differences in location of the common antigen in the respective antigenic patterns of *H. capsulatum* and *B. dermatitidis* and/or a greater immune response in experimentally infected animals to the former agent as shown by the greater immune response of rabbits to histoplasma antigen.

*Experiment 1*—In the course of this study a report by Salvin<sup>17</sup> appeared describing the use of a yeast phase antigen of *H. capsulatum* in the Bengston complement fixation test.<sup>18</sup> No cross reactions between *H. capsulatum* and *B. dermatitidis* were noted, and details concerning titers or degrees of fixation were lacking. Since our technique differs in several fundamental respects from these methods *H. capsulatum* and *B. dermatitidis* antigens were prepared according to Salvin's method and a comparison was made of the two types of complement fixation tests.

Table VI shows the results obtained when the complement fixation was done using Salvin's method in its entirety except that hemolysis was read in per cent. Using the G 2 histoplasma antigen and designating complete hemolysis

TABLE VI BENGSSON'S COMPLEMENT FIXATION TEST UTILIZING THE G 6 HISTOPLASMA ANTIGEN PREPARED ACCORDING TO SALVIN'S METHOD

SERIA	SERUM DILUTIONS (%)					CONTROLS (%)		
	1 10	1 20	1 40	1 80	1 160	ANTIGEN	COMPLEMENT	CELLS
G 2	45	90	95	80	95	100	100	0
G 5	90	90	90	95	95	100		
G 6	90	90	95	95	95	100		
A 1	95	95	95	100	100	100		
A 5	95	95	95	100	100	100		

See footnotes to Table III

sis as the end point, titers of 1 160 were obtained with all three strains of histoplasma antiserum, while the blastomyces antisera against the A 1 and A 5 strains both yielded titers of 1 40. The degrees of hemolysis, however, between two-fold dilutions did not vary appreciably. This was particularly true with the G-5, G 6, A-1, and A-5 antisera where the hemolytic range was limited between 90 and 100 per cent. Two factors probably are involved in this type of reaction (1) excess of complement, two full units used in this test represent approximately 5 1 50 per cent units as calculated by means of the Von Krogh alternation formula<sup>19</sup> taking a mean value of 0 175 for the constant  $1/n$ , this represents almost double the amount of complement used in our test (3 50 per cent units), (2) incomplete fixation at the short one-hour incubation period.

TABLE VII COMPARATIVE EFFECTS OF THE INCUBATION PERIOD ON COMPLEMENT FIXATION, SET A INCUBATED AT 37° C FOR ONE HOUR, SET B INCUBATED OVERNIGHT AT 3 TO 6° C, G 6 ANTIGEN USED IN BOTH SETS

SET A—FIXATION AT 37° C FOR ONE HOUR								
SERA	SERUM DILUTIONS (%)					CONTROLS (%)		
	1 10	1 20	1 40	1 80	1 160	ANTIGEN	COMPLEMENT	CELLS
G 2	0	10	30	60	100	100	100	0
G 5	10	30	80	90	100	100		
G 6	30	50	50	100	100	100		
A 1	95	95	95	100	100	100		
A 5	95	100	100	100	100	100		
SET B—FIXATION OVERNIGHT AT 3 TO 6° C								
G 2	0	0	0	15	50	100	100	0
G-5	20	0	10	40	65	100		
G 6	0	0	5	60	90	100		
A 1	100	100	100	100	100	100		
A 5	100	100	100	100	100	100		

See footnotes to Table III

To evaluate further the second factor, that is the incubation period, two parallel series, A and B, were set up using the G 6 antigen and G 2, G 5, G 6 A-1, and A-5 antisera in the manner used routinely in our test. As noted in Table VII, fixation of set A was carried out at 37° C for one hour while set B was incubated overnight at 3 to 6° C. Definitely stronger and more clear cut fixation was obtained in the set incubated in the refrigerator overnight.

## DISCUSSION

The development and use of a stable specific yeast phase antigen of *H. capsulatum* in complement fixation studies using immune rabbit sera is herein

described. Rabbits immunized with yeast phase *H. capsulatum* antigens developed complement fixing antibodies in high titer reacting with homologous and heterologous strains of histoplasma. No fixation occurred when antisera against *C. albicans*, *C. neoformans*, *S. schenckii*, and *B. brasiliensis* were used. However, antisera against *B. dermatitidis* having a specific titer of 1:40 fixed complement in the presence of histoplasma antigens in low dilutions of serum. When blastomycetes antigens were used, however, the cross reactions observed to exist only in the smaller dilutions between histoplasma and blastomycetes antisera were more pronounced. In fact, because of the weaker immunizing capacity of the blastomycetes antigen as compared with the histoplasma antigen, comparable or even higher titers were observed with the histoplasma antisera. However, these titers of histoplasma antisera against blastomycetes antigen were usually one fourth to one half of the titer obtained when histoplasma antigen was used. For example, histoplasma sera with a titer of 1:160 fixed complement in the presence of the blastomycetes antigen up to dilutions of 1:40. Although numerically this was equivalent to the 1:40 titer of the specific blastomycetes antisera, the latter represented the maximum specific titer obtainable under the conditions of our test. This suggests the presence of a common cross reacting antigen in both *B. dermatitidis* and *H. capsulatum* which confirms the observations made on the basis of skin tests in infected animals by Howell<sup>1</sup> and in complement fixation tests by Tenenberg and Howell<sup>16</sup> and Bunnell and Furcolow<sup>6</sup> in which filtrates of the mycelial phase of histoplasma and blastomycetes were used as antigens. The results of the complement fixation tests can be more accurately evaluated by the joint use of properly prepared *H. capsulatum* and *B. dermatitidis* antigens and serial dilutions of the test sera.

The yeast phase antigen of *H. capsulatum* adapts itself readily to use in the complement fixation test of Kent and Rein<sup>10</sup> in which excess of complement is avoided, adequate fixation is allowed and the per cent hemolysis is utilized to obtain clear cut, specific results. We were unable to confirm Salvin's findings that no cross exists between *H. capsulatum* and *B. dermatitidis* in the yeast phase either by use of the Bengston complement fixation test<sup>17</sup> or by that employed in this laboratory. Our observations are more in accord with others<sup>6, 16, 18</sup> that immunologic cross reactions between these two agents do exist. This would imply that in complement fixation tests for either human histoplasmosis or blastomycosis both antigens should be employed.

#### SUMMARY

A method employing yeast phase antigens of *H. capsulatum* in complement fixation tests with immune rabbit sera is described.

The Kent and Rein complement fixation test utilizing per cent hemolysis was clear cut and definite.

An immunologic relationship between *H. capsulatum* and *B. dermatitidis* was demonstrated.

The potential value of employing yeast phase antigens in complement fixation tests for the diagnosis of past or present infections with histoplasmosis

as a possible adjunct to the present methods now available is open to investigation

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# THE CONCENTRATION OF THE LABILE FACTOR OF THE PROTHROMBIN COMPLEX IN HUMAN, DOG, AND RABBIT BLOOD ITS SIGNIFICANCE IN THE DETERMINATION OF PROTHROMBIN ACTIVITY

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THE prothrombin time, which may be defined as the coagulation time of plasma to which an excess of thromboplastin has been added is generally accepted as a reliable quantitative measure of prothrombin activity. Until recently prothrombin was considered a unitary substance and any diminution of prothrombin activity was interpreted as a specific decrease of this clotting agent. In 1943 one of us (A. J. Q.) found that the loss of prothrombin activity in stored plasma was not due to a diminution of the classic prothrombin which is adsorbable by  $Al(OH)_3$  and which diminishes in dicumarol poisoning but to that of a new factor not previously recognized.<sup>1</sup> This substance was named component A of prothrombin but when it was later found that the adsorbable fraction of prothrombin contained two components, the terms components A and B were applied to these and the substance which disappears in stored plasma was redesignated the labile factor.

Soon after these studies were published Owren<sup>2</sup> independently discovered a similar clotting agent which he named factor V. He found that this factor was lacking in a patient with a serious hemorrhagic diathesis. In retrospect it seems probable that the patient of Rhoads and Fitz Hugh,<sup>3</sup> diagnosed as having idiopathic hypoprothrombinemia, likewise suffered from a lack of the labile factor. Since diminution of this agent causes marked impairment of coagulation and a concomitant bleeding condition it seems obvious that the labile factor is indispensable for coagulation or more specifically for prothrombin activity.

It becomes of great practical importance to know how this new factor affects the prothrombin determination. In this study therefore an attempt has been made to obtain information on that question. So far no means have been found to alter the concentration of this agent in vivo and clinical study too is limited since Owren's case is the only one known at present in which the factor appeared diminished. Stored plasma from which the labile factor has been markedly reduced is the only available means for the assay of the substance in various types of plasma and for studying quantitatively its effect on the prothrombin time. Since human blood contains a relatively low concentration of the labile factor storage will bring about a prompt and striking depletion

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and therefore aged oxalated human plasma was found satisfactory for the present studies. Storage of plasma in open test tubes at ordinary refrigerator temperatures ( $4^{\circ}\text{C}$ ) for seven days usually resulted in an increase of the prothrombin time from a normal of 12 seconds to 40 or more. No attempt was made to isolate the labile factor since it was not considered essential for the objective of the present study. By treating the plasma to be assayed with tricalcium phosphate, both components A and B are removed, thus leaving, as the only known plasma constituents playing a role in the process of clotting, fibrinogen and the labile factor.

#### MATERIAL AND METHODS

*The Concentration of the Labile Factor in Human, Dog, and Rabbit Plasma*—To assay blood for its content of labile factor, it was collected by venipuncture and nine volumes immediately, were mixed with one volume of 0.1M sodium oxalate. The plasma was treated with tricalcium phosphate\* as follows. 1 cc of a 0.008M suspension of tricalcium phosphate was transferred to a small test tube and centrifuged. The supernatant water was poured off and the tube dried. One cubic centimeter of plasma was then mixed with the packed tricalcium phosphate. After ten minutes of incubation at room temperature the adsorbent was separated by centrifugation. The plasma obtained no longer clotted when mixed with thromboplastin and calcium chloride. By this procedure components A and B are removed, but the concentration of the labile factor is only minimally affected, as determined by preliminary studies.

On adding this treated plasma to stored human plasma, the prothrombin time is strikingly shortened. By determining the amount of plasma that has to

TABLE I THE EFFECT ON THE PROTHROMBIN TIME RESULTING FROM THE ADDITION OF VARYING AMOUNTS OF THE LABILE FACTOR (PRESENT IN FRESH AND STORED PLASMA) TO STORED HUMAN PLASMA

Volume of stored human plasma Volume of plasma assayed for labile factor *	100	99	8	99	98	90	80	70	60	50	40	30	20	10
		0.2	1	2	10	20	30	40	50	60	70	80	90	
	PROTHROMBIN TIME (SEC)													
Rabbit, fresh	71	20	15	10½	8½	8	7	7	7	7	8	9	11½	
Dog, fresh	71		20	16	11	8½	8½	8½	8½	8½	8½	9½	14	
Human, fresh	71				21	15½	14	13	12	12	12	13	20	
Rabbit, stored (17 days)†	71		19		13	11	10	9½	9½	9½	9½	11	15	
Dog, stored (17 days)†	71				22	17½	17	16	15	15	15	17	24	
Human, stored (6 days)†	71				27	27½	29	30	33	35	37	44	67	

\*The plasma was treated with  $\text{Ca}_3(\text{PO}_4)_2$  to remove components A and B.

†The prothrombin times of the stored plasma were: rabbit plasma 10 sec., dog plasma 22 sec. and human plasma 26 seconds.

\*To prepare tricalcium phosphate suspension. 158 Gm of trisodium phosphate are dissolved in 1000 cc of distilled water. Separately 66.6 Gm of anhydrous calcium chloride are dissolved in 1000 cc of distilled water. The calcium chloride solution is poured into the trisodium phosphate with thorough stirring. The pH is adjusted to 7. The precipitated tricalcium phosphate is washed repeatedly by decantation until the sodium chloride is removed. The volume is brought to 1 L, thus making a 0.2M suspension. From this stock a 0.008M solution is prepared by mixing 4 cc with 96 cc of distilled water.



be added to a fixed amount of stored plasma in order to reduce the prothrombin time to an arbitrarily selected value, the relative concentration of the labile factor can be calculated. The value taken as standard was 20 seconds, the prothrombin time which is obtained when fresh oxalated human plasma treated with tricalcium phosphite is added to stored oxalated human plasma in the proportion of 1 to 10. It can be seen from Table I that only one part of rabbit plasma needs to be mixed with 500 parts of stored plasma to reduce the prothrombin time to 20 seconds while dog plasma will bring about the same reduction in a dilution of 1 to 100. These findings indicate that fresh rabbit blood contains fifty times and dog blood ten times as much labile factor as human blood.

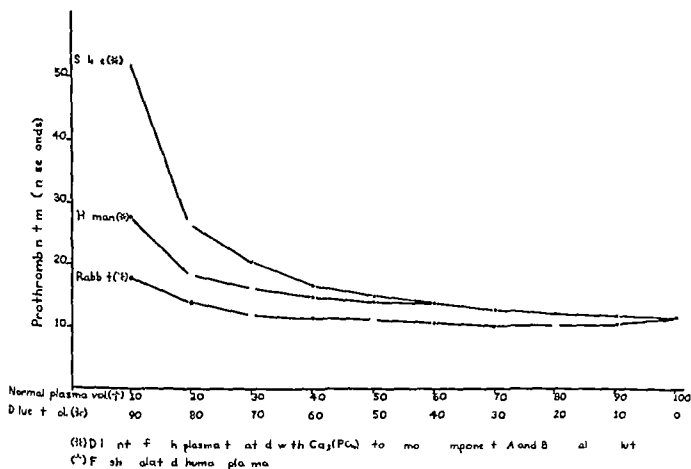


Fig 1—The influence of varying concentrations of the labile factor on the prothrombin time of fresh normal human plasma

When rabbit blood was stored for seventeen days the labile factor decreased to such an extent that a dilution of 1 to 50 was required to obtain a prothrombin time of 19 seconds. Apparently the labile factor decreased approximately to one tenth of its original concentration in seventeen days of storage. A similar decrease occurred in stored dog plasma. Due to the initial high concentration in rabbit and dog plasma the effect of the loss of labile factor in storage as indicated by an increase in prothrombin time is not so striking as in human plasma.

It will be observed that as the volume of fresh plasma treated with tricalcium phosphite added to stored plasma is increased the prothrombin time decreases to a minimal value that remains constant over a rather wide range (Table I). Munro and Munro reported similar findings. The higher the concentration of the labile factor, the lower the minimal constant value. Thus

rabbit plasma reduces the prothrombin time of stored oxalated human plasma to 7 seconds, whereas human plasma lowers it only to 12 seconds

*The Effect of Different Concentrations of the Labile Factor on the Prothrombin Time of Fresh Human Plasma*—In 1945 one of us (A J Q) observed that the prothrombin time of fresh human plasma diluted with progressively increasing amounts of rabbit plasma treated with aluminum hydroxide did not give a typical hyperbolic curve such as is obtained by dilution with saline, but one in which a minimal value was quickly attained and which remained unchanged over a wide range of dilutions.<sup>6</sup> Prothrombin curves obtained by using saline or fresh human or rabbit plasma treated with tricalcium phosphate as the diluent of fresh oxalated human plasma are shown in Fig 1. It seems definite that the high concentration of the labile factor was responsible for the shape of the curve obtained with rabbit plasma, because when fresh human plasma treated with tricalcium phosphate was employed as diluent, thereby keeping the concentration of the labile factor low and constant, the curve approximated that obtained with saline. It is not possible to explain the action of the labile factor on the shape of the curves, but it is obvious that the factor is a key substance in the formation of thrombin.

#### DISCUSSION

One of the most striking observations recorded in this study is the low concentration of the labile factor in human blood. It is certain that this accounts in part for the relatively long prothrombin time of human plasma as compared with that of many of the common laboratory animals such as the rabbit and the dog. But there must be other factors, as shown by the result of the simple experiment of mixing oxalated human plasma with an equal volume of fresh rabbit plasma treated with tricalcium phosphate to remove component A and B but not the labile factor nor fibrinogen. In this mixture the concentration of the labile factor was increased about twenty-five fold, yet the prothrombin time was decreased only from 12 to 10 seconds. If rabbit plasma is diluted with an equal volume of fresh human tricalcium phosphate treated plasma, which produces a mixture having about the same concentration of labile factor, a prothrombin time of 7 seconds is obtained. Since the prothrombin times are not a linear function of prothrombin activity but vary according to the well known hyperbolic curve, and since these values of prothrombin time are in the steep part of the curve, the difference between 10 and 7 seconds is much greater than would be shown by the simple arithmetic ratio.

Studies on the concentration of the labile factor in healthy subjects show that it is remarkably constant. No means so far has been found to alter specifically and exclusively its concentration. It still remains to be determined whether its level is altered in certain pathologic conditions. In the well recognized types of hypoprothrombinemia such as vitamin K deficiency, dicumaiol poisoning, and hereditary deficiency of either component A or B, no significant change in the concentration of the labile factor occurs. In recent years cases of hyperprothrombinemia have been reported. Many of these are based on prothrombin time determinations of highly diluted plasma, often with complete

disregard of the fibrinogen concentration, which as Deutsch and Geisler have shown, can definitely influence the prothrombin time of highly diluted plasma. Most of these cases can probably be dismissed as insignificant but authenticated cases deserve reinvestigation to determine what factor of the prothrombin complex is responsible.

The disappearance of the labile factor in plasma when stored is more easily detected in human than in dog or rabbit plasma because its concentration is relatively low. Oxidation appears to be responsible for the inactivation of the labile factor since plasma covered with a layer of mineral oil does not show nearly as rapid a decrease of this agent. Its instability appears to be associated with decalcification. Thus native hemophilic plasma shows a much slower decrease of the labile factor than oxalated or decalcified hemophilic plasma.<sup>8</sup> Mechanism of action and relationship of this new factor with the other clotting agents require further investigation. The complexity of the problem is plainly shown by the results in Table I.

In addition to the decrease of the labile factor it is obvious that another change occurs in stored plasma. Thus whereas mixing fresh human plasma with an equal volume of rabbit plasma treated with tricalcium phosphate reduces the prothrombin time only to 10 seconds the same mixture with stored instead of fresh human plasma will have a prothrombin time of 7 seconds. Something obviously happens in stored plasma which increases the activity of the labile factor. Whether this is due to the liberation of a specific substance on which the activity of the labile factor depends remains to be determined. It is interesting to speculate if this has any relation to the A $\epsilon$  globulin of Seegers and associates<sup>9</sup> especially since they have obtained evidence that one form the plasma A $\epsilon$  globulin, is converted to a more active modification which they named serum A $\epsilon$  globulin.<sup>10</sup> It should be stated that the labile factor is not A $\epsilon$  globulin, since agents such as tricalcium phosphate adsorb the latter but not the labile factor. It is difficult to correlate the present observations with the investigations on A $\epsilon$  globulin, since in those studies the influence of the labile factor is ignored and therefore not controlled. Its absence in the reaction mixture is not ruled out however since lung thromboplastin which was employed may contain an appreciable quantity as a contaminant.<sup>11</sup>

According to the most recent reports of Ware and Seegers<sup>12</sup> they still hold the view that their purified prothrombin is converted to thrombin solely by thromboplastin and calcium and that their A $\epsilon$  globulin acts merely as an accelerator. This view is not in harmony with the earlier findings of Mertz, Seegers and Smith<sup>13</sup> and of Quick<sup>14</sup> that the conversion of prothrombin to thrombin is a stoichiometric reaction. It seems fairly certain that in this reaction the labile factor is indispensable. Owen<sup>15</sup> voices a similar opinion. "In 1944 and 1945 I was able to show that without factor V no thrombin is formed."

With the discovery of clotting factors other than the classic four of the Morawitz and Fuld and Spiro theory, it becomes exceedingly important to inquire how the accuracy and reliability of the prothrombin time determination are

affected. Perhaps it is best at this time, when the whole problem of coagulation seems to be entering into a new phase of development, for the sake of simplicity to view the process as involving two major groups of factors: first, the factors that constitute the prothrombin complex, second, thromboplastin and substances related to its activation. If the prothrombin time as determined by the one-stage method is normal, one can immediately conclude that the factors constituting the prothrombin complex are normal and that the defect is in the thromboplastin or in its activation. Known conditions of the latter class are hemophilia, acquired hemophilia-like disease,<sup>17</sup> and hemophilia like disease of swine.<sup>18</sup> Hypoprothrombinemia becomes then a generic term including congenital deficiencies of components A and B and the labile factor as well as the various types of acquired conditions in which the prothrombin time is prolonged.

Neither the one-stage nor the two-stage method can be any longer considered specific for the determination of any one constituent of the prothrombin complex. It is clear that the prothrombin time of the one-stage method is a resultant of three distinct constituents, A, B, and the labile factor, and there may actually be other agents not yet recognized. The prothrombin time is therefore essentially a measure of prothrombin activity. The one-stage test has the advantage that it is performed on plasma in which physicochemical and chemical changes have been kept at a minimum. Since it has been demonstrated that the prothrombin time of native (undecalcified) and oxalated plasma is essentially the same,<sup>6</sup> the only major alteration of the blood in the one-stage method, namely the removal of calcium by oxalate, does not cause any fundamental disturbance. The thromboplastin employed, a saline extract of rabbit brain dehydrated with acetone, while essentially a crude preparation, has the advantage that it is almost entirely free of the labile factor and other constituents of the prothrombin complex. This is because rabbit brain can be obtained almost completely bloodless.

The determination of prothrombin activity in terms of prothrombin time of the one-stage method is valuable because evidence is accumulating both experimentally and clinically showing that it correlates with hemostatic effectiveness. Thus a prothrombin time of about 20 seconds has been found to mark the beginning of the bleeding tendency whether the deficiency is due to decrease of component B from dicumarol poisoning<sup>19</sup> or from congenital lack of component A.<sup>20</sup> It is becoming apparent that the one-stage method is a delicate and sensitive test that requires meticulous attention to details. The only satisfactory thromboplastin is rabbit brain dehydrated and prepared exactly as originally outlined by one of us (A. J. Q.). Since changes occur in plasma during storage which affect the prothrombin time, the determination should be done on plasma within one or two hours after collection.

The two-stage method of Warner, Brinkhous, and Smith likewise will require reinvestigation to determine the impact of the new prothrombin factors. The use of dehydrated lung extract as thromboplastin needs critical examination since this product is likely to contain the labile factor as a contaminant.

Likewise there is danger that even the fibrinogen employed may contain the labile factor as an impurity. This probably explains why the two stage method failed to disclose loss of prothrombin activity in stored plasma and led Loomis and Seegers<sup>21</sup> into the errors of attributing the delayed prothrombin time of stored plasma to an alteration of fibrinogen and completely overlooking the true cause, namely the loss of the labile factor.

#### SUMMARY

The labile factor of the prothrombin complex is not adsorbed by tricalcium phosphate and similar adsorbents. It disappears from stored oxalated plasma due to destruction by oxidation and this accounts for the decrease of prothrombin activity in stored plasma.

A simple method for assaying the labile factor in plasma was developed. The plasma to be assayed is treated with tricalcium phosphate and then the amount is determined which must be added to a fixed quantity of stored human plasma to reduce the prothrombin time to an arbitrarily selected value. By this procedure it was found that rabbit plasma contains fifty times and dog plasma ten times as much labile factor as human plasma.

The prothrombin time is reduced to a markedly shorter value when the labile factor is added to stored plasma than when added to fresh plasma, thus suggesting that something is elaborated in stored plasma which increases the activity of the labile factor.

The influence of the labile factor on the accuracy of the prothrombin activity determinations and its variations in physiologic and pathologic conditions are discussed.

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# BLOOD AND BONE MARROW CONCENTRATION OF ATABRINE AND ITS ROLE IN APLASTIC ANEMIA

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THE value of Atabrine in the suppression and treatment of malaria during the recent war cannot be overestimated. Despite the large number of troops to whom the drug was regularly administered very few signs of toxicity appeared. Most prominent were the cases of Atabrine dermatitis complex. The most severe reactions caused by Atabrine therapy were in the cases of aplastic anemia reported by Custer.<sup>1</sup> He described fifty-seven fatal cases in troops from the Asiatic Pacific Theater. This was the first comprehensive study indicating that Atabrine affected the hematopoietic system. Previously Shannon and co-workers<sup>2</sup> showed that the Atabrine concentration in leucocytes was much higher than in plasma or erythrocytes. It might be inferred from this observation that since Atabrine is concentrated in the leucocytes it might act as a toxic agent on these cells and perhaps aid in the pathogenesis of aplastic anemia.

Aplastic anemia primarily involves the bone marrow and it therefore was of interest to determine the concentration and the rapidity of elimination of Atabrine from the hematopoietic organs. In aplastic anemia the myeloid leucocytes are much more severely affected than are the lymphocytic leucocytes and information was needed as to whether Atabrine entered both these cells in comparable concentrations.

This report presents the results of a systematic study of the Atabrine concentration in bone marrow and in various blood elements of the rabbit and chicken and the rate of excretion. These results are correlated with some observations in man.

## EXPERIMENTAL

*Rabbit Experiments*—The concentration of Atabrine in the bone marrow and blood of rabbits was determined at various intervals after a single injection of the drug. Rib marrow levels were taken as representative of those in flat bones, and femoral marrow was analyzed for levels in long bones. Liver, spleen, thymus and mesenteric lymph nodes were analyzed for comparison of visceral levels with marrow levels.

*Method* Each of twenty-one albino rabbits weighing about 2.5 kilograms was injected with a single intramuscular dose of 8 mg per kilogram of Atabrine dihydrochloride. The animals were then sacrificed at intervals of from four hours to twenty-six days. Three animals were studied in each group and the results were averaged. The blood was collected in potassium oxalate and centri-

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fused to separate the plasma and red cells from the buffy coat containing the white cells. All specimens of tissue and blood were analyzed in duplicate using Masen's photofluorimetric method.<sup>3</sup>

**Results** Table I shows the concentration of Atabrine in the tissues studied. After four hours the spleen contained 40.6 mg per kilogram as compared with 7.7 mg per kilogram in the liver. The bone marrow Atabrine varied between 3.1 and 5 mg per kilogram, indicating that there was only a slight difference, if any, between the Atabrine level in flat bone marrow and in long bone marrow. The concentration of the drug in leucocytes was much greater than that found in either plasma or erythrocytes.

TABLE I ATABRINE CONCENTRATION (MG/KG) IN TISSUES OF RABBITS AFTER AN INTRA MUSCULAR INJECTION OF 8 MG/KG OF ATABRINE DIHYDROCHLORIDE

	TIME AFTER INJECTION						
	4 HOURS	4 DAYS	7 DAYS	11 DAYS	13 DAYS	18 DAYS	26 DAYS
Rib marrow	5.08	0.59	0.37	Trace	0	0	0
Proximal femoral marrow	4.38	1.02	0.33	Trace	0	0	0
Distal femoral marrow	3.11	0.69	0.21	Trace	0	0	0
Plasma	0.08	Trace	0	0	0	0	0
Erythrocytes	0.27	Trace	0	0	0	0	0
Leucocytes	1.87	Trace	0	0	0	0	0
Lymph node	1.70	1.35	1.05	0.41	0.19	0.29	0.19
Thymus	2.70	0.57	0.10	0	0	0	0
Liver	7.78	1.25	0.46	0.05	0.11	0	0
Spleen	40.62	1.96	0.44	0.11	0.14	0	0

Four days after the administration of Atabrine the spleen still had the greatest concentration of drug, but this level had dropped more precipitately than had any of the others. Despite a significant level in the bone marrow, the peripheral erythrocytes, leucocytes, and plasma were practically free of Atabrine. During the second week after injection the levels in all tissues decreased rapidly. The thymus contained no Atabrine on the eleventh day, where as the lymph nodes contained the drug as late as twenty six days after injection.

**Chicken Experiments**—Chickens are frequently utilized in studying the effects of antimalarial drugs, and therefore experiments were conducted to determine the concentration of Atabrine in the marrow and blood elements of this animal. Liver and spleen again were analyzed for comparison. Since chicken erythrocytes are nucleated, the comparative concentration of Atabrine in the leucocytes and erythrocytes was of interest.

**Method** Eight Leghorn hens weighing about 1.7 kilograms each were injected with single intramuscular doses of 8 mg per kilogram of Atabrine dihydrochloride. They were sacrificed at intervals of four hours and four, seven and ten days. Two birds were examined at each period. The tissues were treated as described in the rabbit experiments.

**Results** In general, at four hours the Atabrine concentrations in the chicken tissues were similar to those found in rabbits at a corresponding time after injection. The same relationship also existed between the various blood components, in that the erythrocyte concentration of Atabrine was higher than that of the plasma but much lower than that of the leucocytes (Table II).



TABLE II ATABRINE CONCENTRATION (MG/KG) IN TISSUES OF CHICKENS AFTER AN INTRA MUSCULAR INJECTION OF 8 MG/KG OF ATABRINE DIHYDROCHLORIDE

	TIME AFTER INJECTION			
	1 HOUR	4 DAYS	7 DAYS	10 DAYS
Proximal femoral marrow	5.08	0	0	0
Distal femoral marrow	6.11	0	0	0
Plasma	0.17	0	0	0
Erythrocytes	0.91	0	0	0
Leucocytes	3.32	0	0	0
Liver	21.64	0.21	0.07	0.04
Spleen	15.82	0.10	0.04	0.05

In the case of the chickens however the concentration of Atabrine in the marrow was reduced to zero within the first four days whereas this result was obtained only after thirteen days in the rabbits receiving a comparable dose of the drug. Another species difference was found in the splenic concentrations of Atabrine at four hours. In the rabbit the level in the spleen was much higher than that of the liver whereas in the chicken the reverse was found.

*Observations on Human Beings*—The purpose of these experiments was to compare the levels of Atabrine in the bone marrow with those found in the peripheral blood. By utilizing patients with either myelocytic or lymphocytic leucemia it was also determined whether Atabrine was found in similar concentrations in both lymphocytic and myelocytic leucocytes.

*Method* Two male subjects one normal and the other with a recurrence of symptoms from infection with vivax malaria, received Atabrine by mouth. Twenty-four hours after completion of a course of 2.4 Gm. of Atabrine samples of peripheral blood and aspirated bone marrow were obtained. The bone marrow in these instances was markedly diluted with peripheral blood as compared with the bone marrow removed from rabbits after autopsy. Three additional subjects two with myelocytic leucemia and one with lymphocytic leucemia were given Atabrine orally for varying periods of time. The bone marrow and peripheral blood of three patients and the peripheral blood of the other two were studied.

TABLE III BLOOD AND BONE MARROW CONCENTRATION AFTER ORAL ADMINISTRATION OF ATABRINE DIHYDROCHLORIDE TO FIVE PATIENTS

PATIENT	CONDITION	LEUCOCYTES PER MM <sup>3</sup>	ATABRINE LEVEL ( $\mu\text{G} \times \text{LITER}$ )			
			PLASMA	ERYTHROCYTES	LEUCOCYTES	BONE MARROW
1	Malaria	7,000	42	-	-	836
2	Normal	5,500	77	55	-	1200
3	Chronic myelocytic leucemia	75,000	154	200	1285	2381
4	Chronic myelocytic leucemia	75,000	250	392	1589	-
5	Chronic lymphocytic leucemia	90,000	53	57	1864	-

Doses varied so that absolute levels between patients are not comparable.

*Results* As in the rabbit experiments it was found that the bone marrow had a higher concentration of Atabrine than either the plasma or erythrocytes (Table III). The marrow concentration was as much as twenty times that of the plasma despite the fact that the marrow was mixed with peripheral blood.

The patients with leucemia, whose leucocytes were mainly myelocytic or lymphocytic cells, showed high concentrations of Atabrine in their white cells. It was thus determined that lymphocytes also had a high concentration of Atabrine similar to that found in myelocytic cells.

#### DISCUSSION

There have been but few reports dealing with Atabrine in the various blood elements and in bone marrow. Histologically, Martin and co-workers<sup>4</sup> noted deposition of the drug in various tissues although the blood and bone marrow were not specifically mentioned. Siegel and Mushett<sup>5</sup> reported that after large doses of Atabrine the bone marrow of rats showed an increase in the number of segmented and nonsegmented neutrophils. Reticulo endothelial cells containing vacuoles and basophilic inclusions occasionally were found. Significant levels in the bone marrow of chickens have been noted for at least two days after a single intravenous injection of Atabrine.<sup>6</sup> Rather high levels were reported in rabbit marrow six days after a large oral dose.<sup>7</sup> The present study confirms the species difference in that Atabrine remains in the bone marrow of rabbits longer than it does in the marrow of chickens. This species difference is mentioned because of the frequent use of chickens in the study of malaria and its treatment.

This investigation revealed that although Atabrine was found in the marrow of rabbits in high concentrations, the rate of disappearance of the drug from the marrow was not markedly different from that found in other tissues. Thus eleven days after a single dose of Atabrine the bone marrow as well as other tissues showed only very small amounts of the drug present. This level soon reached zero, indicating that marrow does not retain Atabrine for long periods of time. Two weeks after completion of the Atabrine course, in one of the leucemic patients the bone marrow was free of the drug. No studies for the detection of Atabrine degradation products were undertaken.

The British Army Malaria Research Unit<sup>8</sup> showed that when Atabrine was given to patients with myelocytic leukemia the whole blood concentration rose as the white cell count rose. The present study, utilizing leucemic patients and analyzing the leucocytes directly, reveals that the lymphocytes as well as the myelocytic cells concentrate Atabrine in large amounts.

The question arose as to whether the ability of leucocytes to concentrate Atabrine and the inability of erythrocytes to do so was due to the fact that the leucocytes contain nuclei. It is recognized that there probably exists a fundamental difference between the nuclei of the red and white cells, nevertheless, in the chicken, in which the peripheral erythrocytes are nucleated, it was found that these cells did not have as high a concentration of Atabrine as did the white cells. From this it seems likely that in the chicken, and probably in other animals, none of the cells of the erythrocytic series take up large quantities of Atabrine and that therefore the high concentration of the drug in bone marrow is chiefly due to the presence of the drug in the myelocytic series of cells.

Although histologically the lymph node and thymus are quite similar and the concentration of Atabrine in both tissues was found to be almost the same at

TABLE IV LACK OF CORRELATION BETWEEN ATABRINE CONCENTRATION IN BLOOD CELLS AND IN TISSUES AND THE FATE OF THESE IN APLASTIC ANEMIA

	MYELOCYTIC LEUCOCYTES	LYMPHO-CYTIC LEUCOCYTES	ERYTHROCYTES	BONE MARROW	LYMPHATIC SYSTEM
Atabrine concentration	High	High	Low	High	High
Affected in aplastic anemia	Yes	No	Yes	Yes	No

four hours the subsequent finding was markedly different. Whereas no Atabrine was detected in the thymus at the end of eleven days the lymph nodes retained the drug for more than twenty six days.

An attempt has been made to correlate the present findings in animals and man. It is believed that the results obtained prove that the alleged relationship between Atabrine and aplastic anemia is not a direct one. It is not due to the fact that the drug enters the blood cells in high concentrations and by acting locally destroys them. Although Atabrine concentrates in both lymphocytic and myelocytic leucocytes, only the latter are affected in the disease. Although the drug does not enter the red cell series in large amounts these cells are severely affected (Table IV). There appears to be no correlation between concentration of the drug in various blood cells and those cells affected in aplastic anemia. Because of these findings and the further fact that of the millions of people who took Atabrine only a few developed aplastic anemia it may be presumed that the role of Atabrine in the pathogenesis of aplastic anemia falls into the idiosyncratic group of responses.

#### SUMMARY

After a single dose of Atabrine injected into rabbits the concentration in the bone marrow was much higher than that in the plasma or erythrocytes. More time was required for complete excretion of the drug from the bone marrow than from the peripheral blood.

Lymphocytic and myelocytic leucocytes both contained Atabrine in relatively high concentrations. Their ability to concentrate the drug is not due to the presence of a nucleus since the nucleated erythrocytes of the chicken do not develop as high a concentration of Atabrine as do the leucocytes.

Aspirated human sternal marrow, although greatly mixed with blood showed a concentration of Atabrine twenty times as great as did the plasma. The mesenteric lymph nodes of the rabbit retained considerable amounts of Atabrine long after the liver and spleen were free of the drug.

There appears to be no correlation between the height of concentration of Atabrine in the various blood cells involved in aplastic anemia and the damage evident in those same cells when aplastic anemia develops. The alleged role of Atabrine in the causation of this disease would appear to be in the nature of an idiosyncrasy.

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# THE EFFECT OF THE LIGATION OF THE PANCREATIC DUCTS AND OF PANCREATECTOMY AFTER DUCT LIGATION ON SERUM LIPASE

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THE LITERATURE is in general agreement that ligation of the pancreatic ducts in dogs leads to an immediate increase of the amylase in the blood. This unity of opinion, however, does not exist regarding the effect of tying of the ducts on the blood lipase. Cherry and Crandall<sup>1</sup> have found an olive oil splitting enzyme in relatively large amounts in the blood of dogs after ligation of the pancreatic ducts. The results have been confirmed in two dogs by Dozzi<sup>2</sup> and in cats by Roe and Goldstein<sup>3</sup> and Goldstein and co-workers.<sup>4</sup> But Popper and Sotter<sup>5</sup> did not observe an increase of blood lipase after the operation. Referring to the experiments of these investigators Braum<sup>6</sup> believes that elevation of blood lipase after tying the pancreatic ducts has not yet been definitely proved.

Through the work of Comfort and Osterberg<sup>7</sup> and Johnson and Bockus<sup>8</sup> the experiments of Cherry and Crandall have become the basis for the clinical use of the serum lipase test in the diagnosis of pancreatic disease. However, if Popper and Sotter's results are correct the serum lipase test is deprived of its essential experimental confirmation.

Since we have been interested in the evaluation of this test and its experimental foundations for years we were confronted with the following questions:

Does the serum lipase rise significantly after ligation of the pancreatic ducts in dogs or not?

If the serum lipase does rise after ligation of the pancreatic ducts in dogs do these increased amounts of serum lipase originate in the pancreas?

Is the pancreas its only source?

What is the probable mechanism for the increase?

We began our investigations with the determination of the serum lipase before and after ligation of all pancreatic ducts on a large scale in order either to confirm Cherry and Crandall's results or to disprove them.

Our findings are based on observations on dogs chiefly female. Animals weighing from 6 to 11 kilograms were used. Those weighing about 20 pounds were preferable since removal of 15 to 20 cc. of blood was necessary for the chemical determinations and on some dogs many of these had to be made. Blood was withdrawn from the heart by puncture or from the jugular vein.

Dogs were fed once or twice a day on a mixture of a prepared (commercial) dog meal, evaporated milk and canned tomatoes with occasional supplements of ground meat when it was available.

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Anesthesia was induced with veterinary Nembutal\* by the combined intravenous and intraperitoneal routes. The optimum method in our hands was to give initially an intravenous injection of about 0.2 cc per kilogram of body weight, after the peritoneal cavity was opened, the anesthetic solution was injected into it as needed.

Simple ligation and division of the pancreatic ducts, total pancreatectomy, partial pancreatectomy, and duct ligation followed by total pancreatectomy after an interval of several days, have been performed in the course of our studies. In the simple ligation and division of the ducts the pancreatic lobules were separated from both the duodenum and the pancreaticoduodenal blood vessels by sharp dissection of the delicate connective tissue adherent to the pancreas and by a gentle peeling action of the bare or gauze covered finger. The ducts were difficult to isolate, but with meticulous care few were missed. Almost always one large duct was fairly easy to identify and ligate. The other ducts usually one, sometimes two, and rarely three, were, with exceptions, proximal to the main duct. These ducts were invariably difficult to locate as they were always short and of fine caliber. In order to diminish chances of the pancreatic juice getting into the intestine and to facilitate healing, the omentum in 12 dogs was secured to the raw surface of the pancreas or interposed between the separated pancreas and duodenum. When ligation and division of the ducts had been done previously, total pancreatectomy proved to be more difficult, due to the intense inflammatory reaction in the region of the duodenum and pancreas, than total removal of the intact normal pancreas.

#### LIPASE METHOD

This method for lipase determination utilizes three blanks for every determination.  $B_1$  represents the serum blank,  $B$  the olive oil blank, and  $B_2$  the buffer blank.

$B_1$	$B$	$B_2$	TEST
2 cc H <sub>2</sub> O	2 cc Oil emulsion	3 cc H <sub>2</sub> O	2 cc Oil emulsion
5 cc Calcium acetate	5 cc Calcium acetate	5 cc Calcium acetate	5 cc Calcium acetate
5 cc Buffer	5 cc Buffer	5 cc Buffer	5 cc Buffer
1 cc Serum	1 cc H <sub>2</sub> O		1 cc Serum

Incubate the four flasks at 37° for twenty-four hours. At the end of incubation add 10 cc of alcohol ether inactivating mixture to each flask. After having added a few drops of phenolphthalein, titrate with N/20 NaOH to a definite pink. Calculation: Add  $B_1$  to  $B$  and subtract  $B_2$ . Finally, subtract this result from the test.

Example	$B_1$ 1.2 cc	$B_1$ 1.2	Test 2.15
	$B_2$ 0.85 cc	$B_2$ +0.85	-1.70
	$B_2$ 0.35 cc	$\hline$ 2.05	0.45 cc
	Test 2.15 cc	$B_2$ - .35	
		$\hline$ 1.70	

#### Reagents —

**Olive Oil Emulsion** Gum acacia, 12.5 Gm., olive oil, 50 cc., H<sub>2</sub>O, 100 cc. Pour the gum acacia and 50 cc of H<sub>2</sub>O into a Waring blender and thoroughly mix. Then add the olive oil slowly and allow to mix to a pure white emulsion. Then add 50 cc more H<sub>2</sub>O and mix to a uniform consistency. Store in the icebox.

\*Abbott Laboratories North Chicago Ill

*Calcium Acetate*  $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot \text{H}_2\text{O}$  Calcium acetate 20 Gm per liter of  $\text{H}_2\text{O}$

*Barbiturate Buffer* Sodium diethylbarbiturate 5 Gm per liter of  $\text{H}_2\text{O}$

*Alcohol Ether Inactivating Mixture* Alcohol 95 per cent 900 cc ether, 100 cc

The results of our experiments concerning the effect of pancreatic duct ligation on serum lipase are shown in Table I and Fig. 1

This enzyme was determined twenty eight times in twenty dogs before the operation. The average amount of serum lipase found in these twenty dogs was 0.6 unit the lowest value was 0.2 the highest values were 1.3 and 1.6 in one dog and 1.0, 1 and 1.2 in three other dogs in all the other animals it was not higher than 0.8. All animals except Dog 20 showed a marked increase of the

TABLE I THE EFFECT OF LIGATION OF THE PANCREATIC DUCTS ON SERUM LIPASE

DOG	SERUM LIPASE BEFORE DUCT LIGATION		SERUM LIPASE AFTER DUCT LIGATION (DAY)										BEYOND THE TENTH DAY
			1	2	3	4	5	6	7	8	9	10	
1	0.6	0.7	9.7	8.2									
2													16.1 (1.44) 24.1 (1.89)
3	1.1				8.4								
5	1.6	1.3			2.3								2.1 (1.41) 10.1 (1.8)
6	0.3							9.9					10.6 (1.6)
10	0.5	0.8	7.3	6.9									
11	0.4			7.5		6.9							
12	0.4			5.3		5.2							
13	1.0			5.0			1.2						
14	0.5				4.4			2.3					
15	0.3					7.9							
16	0.7					2.8							
17	0.6			3.5				3.5		5.8			
18	1.2			10.9				6.7		5.8			
19	0.6							1.1					
20	0.7				1.4			0.8					
21	0.3	0.2	3.6										
22			4.1										
23	0.5	0.2	5.2		6.2								
24	0.5	0.8	4.5		7.2								
25		0.4		6.2									
26	0.2	0.4	4.8						7.8				8.6 (1.1)

serum lipase after the ligation of the pancreatic ducts. This rise begins within twenty four hours after the operation. We determined the serum lipase in six dogs the first day following the ligation and we found values between 3.6 and 7.3. The average amount of serum lipase in these six dogs was 0.4 the average amount twenty four hours after the ligation was 5.1 in other words on the average the values increased thirteen times over the initial values. On the second day after the operation the figures for serum lipase were still rising the average being 7.0. After six or seven days the enzyme seemed to drop in some dogs in others it appeared to rise and in two dogs the highest values were found ten and twenty two days after the ligation. Only Dog 20 showed an insignificant increase of serum lipase from 0.7 to 1.4 on the third day after the operation. It is possible, however that from the fourth to the seventh day when determinations of serum lipase were not performed the values might have been higher.





and dogs or we could produce hyperlipemia by ligating the ducts and then some days later removing the gland. Dozzi, approaching the same problem chose the first method. Utilizing a modification of Cherry and Randall's technique he determined the serum lipase before and after pancreatectomy in a single dog. The values before the operation ranged from 0.1 to 1.15. On the first day after removal of the pancreas the serum lipase was 1.05, on the second day it was 1.7 and on the fifth shortly before the dog died it was 0.6. The experiments of Roe and Goldstein and of Goldstein, Jacobson, Telford and Roe performed on cats are not conclusive either and hence one cannot base a reliable opinion upon them.

TABLE II THE EFFECT OF PANCREATECTOMY ON SERUM LIPASE

DOG	SERUM LIPASE BEFORE PANCREATECTOMY		SERUM LIPASE AFTER PANCREATECTOMY (DAYS)				
			1	2	3	4	5
1	1.0		0.78	0.25	0.4		0.4
27	0.6	0.7	0.6	0.3	0.3	0.1	
28	0.5	0.8	0.6	0.4		0.2	

Total pancreatectomy alone was done in three dogs (Table II) while in five dogs total pancreatectomy was carried out five to nine days following duct ligation (Table III and Fig. 2). As can be seen from Table II the dogs on which pancreatectomy was performed without previous duct ligation showed a steady decrease of serum lipase until death occurred five or six days after the operation. After tying all the pancreatic ducts in two dogs the corpus pancreatis was removed, leaving the processus lienalis and processus uncinatus in situ. Thus from one half to two thirds of the total pancreatic tissue was extirpated. The pancreatic tissue that remained, although able to prevent diabetes, was not sufficient

TABLE III EFFECT OF LIGATION OF THE PANCREATIC DUCTS FOLLOWED BY TOTAL PANCREATECTOMY ON SERUM LIPASE

	DOG 13	DOG 14	DOG 15	DOG 16	DOG 18
<i>Lipase before first operation</i>					
	1.0	0.55	1.0	0.65	1.15
<i>Days after ligation</i>	<i>Lipase after ligation of pancreatic ducts</i>				
2	5.0				10.9
3		4.4			
5			7.85	2.75	
6	1.2	2.25			6.65
9					5.8
<i>Pancreatectomy day after ligation</i>					
	6	6	5	5	9
<i>Days after pancreatectomy</i>	<i>Lipase after pancreatectomy</i>				
2	0.15	0.18	2.35	0.7	
3					0.1
7		0.1			
9		0.55	1.51		
16		1.1			
19			1.9		
20		1.9			
26		1.2			
<i>Death after pancreatectomy (day)</i>					
	5	41	34	6	5

to cause an elevation of the serum lipase. The effect of the removal of the pancreas on the level of serum lipase where the ducts have been ligated previously is clearly indicated in Table III (Fig 2). All five dogs showed a marked rise of the serum lipase after tying of the ducts. (These results are already included in Table I.) After removal of the pancreas there was an immediate drop of

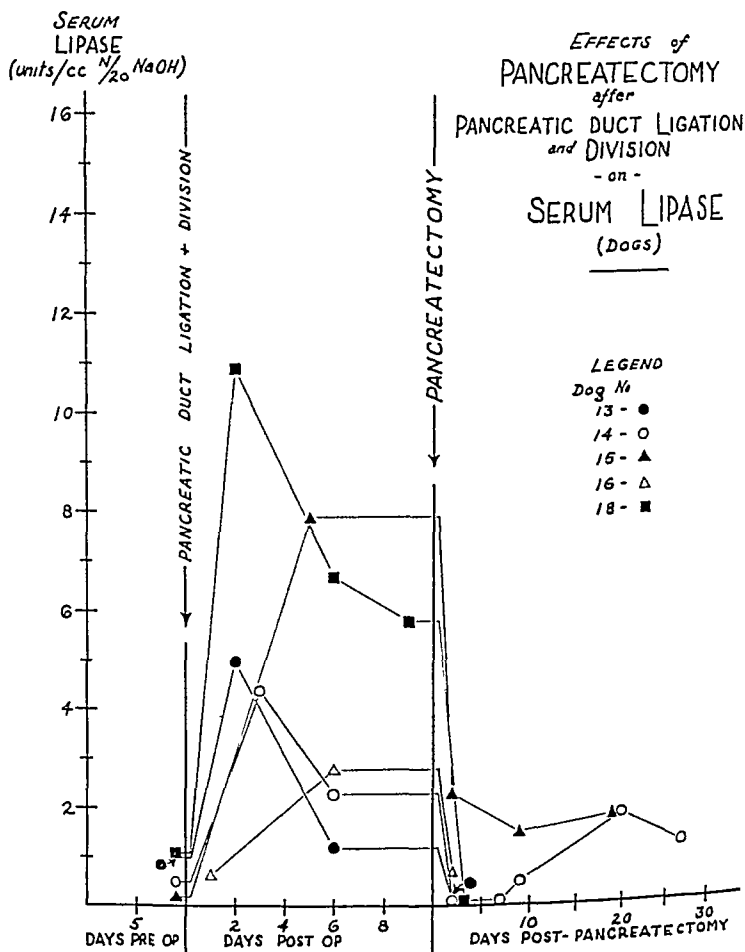


Fig 2

the serum lipase. In three animals (Dogs 13, 14, and 18) the values for the lipase were almost zero on the second day following the operation. In the two remaining animals (Dogs 15 and 16) there was an essential decrease of the enzyme but it was less pronounced than in the other three animals.

Dogs 13, 16, and 18 died a few days after the second operation, but Dogs 14 and 15 were still living three and four weeks after pancreatectomy. We were surprised to find rising figures for serum lipase in these two dogs. One dog showed a value of 1.9 on the nineteenth day after the operation, and the other one showed 1.9 on the twentieth and 1.2 on the twenty-sixth day after the removal of the gland.

Our experiments demonstrate that serum lipase originates at least partly in the pancreas. This is proved by these three facts: (1) Tying of the pancreatic ducts leads to an immediate rise of the serum lipase. (2) Complete pancreatectomy leads to a steady decrease of the enzyme. (3) Total removal of the pancreas at a time when the level of serum lipase is still high, as a result of ligating the ducts, leads to a rapid diminution of the enzyme almost down to zero. We are not able to explain why other authors (Popper and Sotter) did not succeed in producing elevated levels of serum lipase after ligation of the pancreatic ducts, but our findings that two to three weeks after total pancreatectomy serum lipase appears again to increase in amount even to values higher than before operation, show that this enzyme may also be derived from extra-pancreatic sources. The liver is a possible source. In studies concerning the enzymes of the bile in man, we have regularly found lipase in the contents of the gall bladder and the hepatic duct (Unpublished data).

The increase of serum lipase is apparently due to its absorption into the blood stream when the flow of pancreatic juice has been blocked. To substantiate this conclusion we have ligated the pancreatic ducts and drawn the tail of the pancreas through the abdominal wall, exposing the tip through the skin.

TABLE IV THE EFFECT OF LIGATION OF THE PANCREATIC DUCTS FOLLOWED BY THE PERFORMANCE OF A PANCREATIC FISTULA ON SERUM LIPASE

	DOG 24	DOG 27	DOG 28	DOG 29
	LIPASE			
Before operation	0.4	0.9	0.3	0.4
		0.25	0.4	
After ligation of ducts tail drawn through abdominal wall (day)				
1		2.2	3.6	
2	2.0			
3				6.2
4		3.05	6.6	
	Fistula day after ligation			
	3	5	5	4
After fistula (day)				
1				4.0
2	1.7			
3		0.08	2.1	
6	0.5			0.3
8			0.7	
10		0.1		
13				0.6
15			0.5	
18	0.2			
32	0.4			

The serum lipase rose from 0.4 to 2.0 within forty eight hours. We then transected the tip of the exposed gland thereby permitting the pancreatic juice to escape. The first determination of the lipase two days after this operation showed that the lipase was already decreasing. When the fistula was well established, the serum lipase returned to normal, as is shown in Table IV. This demonstrates that the removal of the obstruction to the flow of pancreatic juice by means of a pancreatic fistula after duct ligation allows the escape of the pancreatic juice from the pancreas and therefore prevents the increase of the serum lipase level.

## SUMMARY

Serum lipase is of both pancreatic and extrapancreatic origin. Its pancreatic origin is proved by the following facts: (1) Rise of serum lipase after ligation of the pancreatic ducts in all of twenty-one dogs. (2) Decrease of the serum lipase after total pancreatectomy. (3) Immediate drop of the serum lipase to almost zero in five days after total pancreatectomy, when the level for serum lipase previously has been raised by duct ligation.

The recurrence of serum lipase two to three weeks or longer after total pancreatectomy proves that there exists also an extrapancreatic source for the enzyme.

The mechanism which leads to the rise of serum lipase is absorption of the enzyme into the blood stream after blocking of the flow of the pancreatic juice. When ligation of the ducts is followed by the production of a pancreatic fistula, the elevated serum lipase drops to normal levels.

The authors wish to thank Miss Lillian Rosenberg, Miss Bernice Rubinowitch, and Miss Mary Powers for assistance in the technical determinations.

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## A RESIN ARTIFICIAL KIDNEY

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WASTE products excreted by the kidneys have been removed from the body via artificial routes such as peritoneal irrigations<sup>1, 2</sup> and peritoneal irrigation and gastric lavage,<sup>3</sup> and by in vivo dialysis.<sup>4</sup> The present report is concerned with preliminary observations on another technique by which such removal may be accomplished.

This artificial kidney consists of a resin bed composed of nine parts of Amberlite IR - 100 H\* and one part of Deacidite†. The Amberlite is a typical cation exchange resin with exchange activity on  $-\text{O}-\text{COO}^-$  and  $-\text{SO}_3$  groups. The Deacidite is a typical anion exchange resin with exchange activity on  $\equiv \text{N}(\text{H}_3\text{O})^+$  groups.<sup>5</sup>

### CONSTRUCTION OF RESIN BED

A coarse mesh of each resin is obtained by sifting through a 20 mesh screen. The finer particles require a positive push and care is needed to limit pulverization. Residual fine particles are removed by thorough washes. Deacidite tends to float and remixing is necessary before packing.

The resin mixture is placed in glass columns as in Fig. 1. The column used had an inside diameter of 4 centimeters. Two lengths 50 and 85 cm. have been tested. Rubber stoppers accommodate the inflow and outflow tubes. The outflow tube is curved down and overlaid with glass beads. During packing the glass column is rotated while maintained at a 15 degree angle in order to minimize the formation of direct channels. Additional washes encourage packing and make particle escape through the outflow negligible. The stoppered end is sealed with shellac. A second larger glass tube (outside diameter, 7.5 cm.) is likewise sealed in place about the primary tube and accommodates a temperature control water jacket.

Autoclaving is not feasible as it tends to break the resin particles mainly Deacidite. Sterility is attained with ethyl alcohol. The bed is thoroughly washed with distilled water. At this time the outflow water should give a negative or trace nitrogen test (no resin in solution).

### OBSERVATIONS

One group of in vitro experiments included the following steps: (1) Conditioning of resin bed with Solution P‡; (2) perfusion of resin bed with Solution P containing a high concentration of urea; (3) periodic measurement of the outflow urea concentration; (4) washing with Solution P and measurement of the amount of urea recovered. The volume flow was 100 cc. per minute.

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Technical assistant, Mr. E. A. Lyle.

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Resinous Products Chemical Co., Philadelphia, Pa.

†Permutit Co., New York, N. Y.

‡Solution P of Odel and Ferri<sup>6</sup> has the following composition per liter: NaCl, 6 Gm; KCl, 0 Gm; CaCl<sub>2</sub>, 0.1 Gm; MgCl<sub>2</sub>, 0.1 Gm; NaH<sub>2</sub>PO<sub>4</sub>, 0.05 Gm; NaHCO<sub>3</sub>, 3 Gm. The glucose was lowered to 0 Gm.

Fig 2 illustrates the results. In this experiment the 4 liters of Solution P contained 16 Gm of urea of which 4.78 Gm (30 per cent) were extracted. With this bed, urea concentration, and flow, the urea uptake amounted to 89 per cent at 2 liters. The wash curve mirrored the uptake curve and 4.9 Gm of urea were washed out.

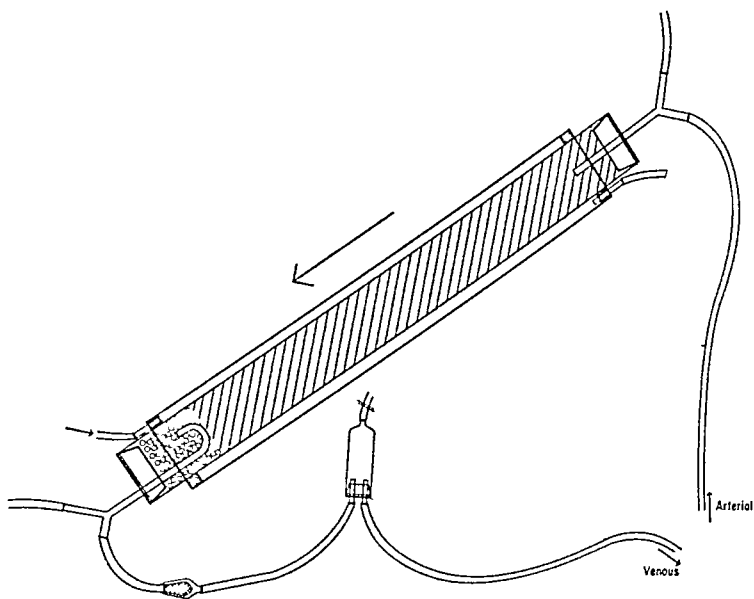


Fig 1—A diagrammatic representation of the resin bed and connections as used in the in vivo perfusions. The description is in the text.

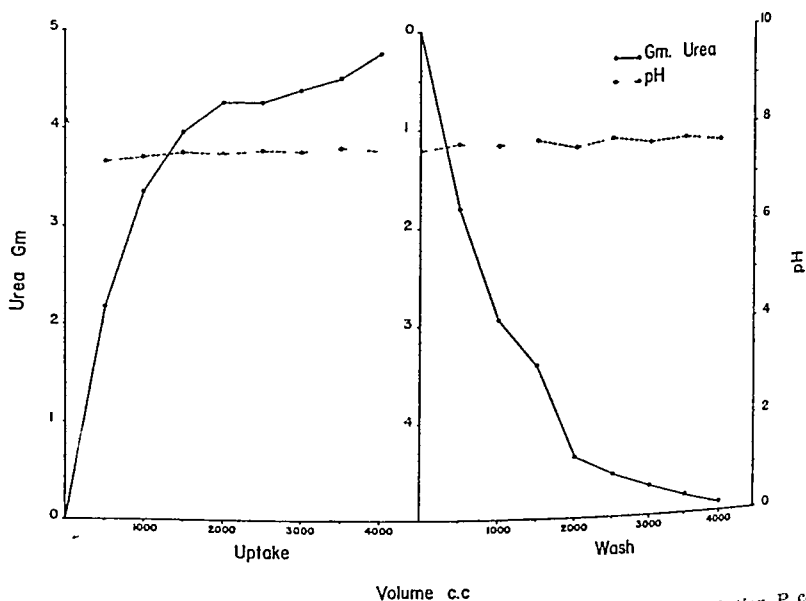


Fig 2—A demonstration of the urea uptake by the resin bed from Solution P containing 100 mg per cent of urea (16 Gm in 4 liters) and the subsequent urea discharge with solution P as wash. Each point represents the cumulative results up to that volume. The steady pH reading (glass electrode meter) is depicted. The following conditions pertained: resin dry weight 600 Gm; diameter of column 4 cm; length of column 80 cm; volume flow 100 cc per minute.

Heparinized blood perfused through the bed conditioned with heparinized Solution P yielded similar results (See Fig. 3)

Six dogs were subjected to *in vivo* perfusion with this apparatus on the fourth day following bilateral nephrectomy. For these experiments the resin column was tilted to a 45 degree angle. The lowest stratum was composed only of Amberlite since the Deacidite goes into solution more readily and was removed by this Amberlite layer. The intake tube was a Y tube, one limb being

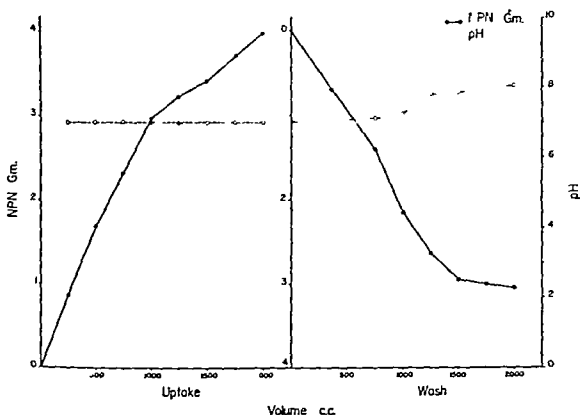


Fig. 3—Observation similar to the one in Fig. 2, heparinized blood used. Urea was added to the blood and the results were gauged as nonprotein nitrogen values. The initial nonprotein nitrogen concentration was 313 mg per cent. The same resin bed was used and the uptake and discharge of urea were approximately twice that in Fig. 2.

connected to the femoral artery and the other to a 20 liter reservoir of heparinized Solution P. One limb of the Y outflow tube was connected to a wash collection reservoir and the other limb was connected to an inverted sylvan graduate acting as an air trap. The air trap led to a fine mesh cloth filter and on to the femoral vein. This filter removed fibrin strands and any resin particles escaping the bed. All connections were with rubber tubing. The distance from artery to intake tube was about 60 centimeters. An interposed manometer recorded the arterial pressure. The volume flow was maintained between 70 and 100 cc per minute.

The resin bed was filled with dog's blood or a substitute (plasma or albumin solution) in order to prevent hypotension. Perfusion was allowed for ten minutes then the blood residuum was collected in the air trap reservoir and the bed was washed with 1000 to 1500 cc of wash. The reservoir blood was reinfused into the vein while the bed was filling again.

Protocol, Dog 6 weight 15 kilograms. *In vivo* perfusion fourth day after bilateral nephrectomy. 50 mg heparin intravenously, wash solution. Solution P plus heparin 50 mg/per 1000 cc, size of bed 500 cc coarse resin bed conditioned with 2000 cc wash. temperature controlled 39° C. number perfusions

4, ten minutes each, flow, 70 c c per minute, total urea removed, 35 Gm (collected for 6 per cent blood contamination), no hemolysis, no reaction, blood pressure average 135 mm Hg, lived 25 more days, total 65 days

#### COMMENT

Preliminary experiments show promise for the resin artificial kidney. In vitro observations demonstrate an efficient means of adjusting pH, maintaining osmolar concentration, adjusting ionic disturbances, and removing waste products. In vivo perfusions have demonstrated a minimal reaction rate and an absence of hemolysis. Waste products are removed but a more efficient means of discharging the bed of residual blood prior to the wash is needed to increase the bulk removal of wastes and to prevent an excessive fluid intake into the recipient. Observations in this direction are being conducted at present.

Reactions to the resin (mainly Deacidite) were observed earlier with insufficient wash and failure to use the final Amberlite layer to remove soluble Deacidite. The reactions included restlessness, muscular jerks, and tachypnea.

Thorough preliminary conditioning of the bed with a heparinized solution is essential to avert clotting. Metal parts are not used since they encourage clotting of heparinized blood.<sup>4</sup>

The efficiency of this technique requires improvement. Translation of this technique to clinical use is not indicated at present.

The resin used for the removal of wastes (Amberlite) was primarily devised for the removal of cations, it does not seem to be as efficient in the removal of nitrogenous wastes and presumably other waste products. Resins more suitable to this purpose seem highly desirable.

#### CONCLUSION

A rather simple technique has been described for the removal of nitrogenous waste products. In vivo experiments have demonstrated minimal reactions, no hemolysis, and a simple means of maintaining pH and osmolar concentration steady. Removal of excessive amounts of cations may be attained by varying the conditioning of the resin.

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THE RELATION OF PHENOL RETENTION TO UREMIA  
AND  
THE EFFECT OF PHTHALYL SULFATHIAZOLE AND STREPTOMYCIN  
ON PHENOL PRODUCTION

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FROM time to time various urinary constituents which are retained during renal insufficiency, have been implicated as primary factors in producing the symptom complex known as uremia. The depression of the central nervous system seen in uremia has been attributed to the accumulation of phenols<sup>1, 2</sup>

The source of the phenolic compounds which accumulate during renal insufficiency has been shown by Marenzi to be the intestinal tract.<sup>3</sup> He demonstrated that, while in nephrectomized animals the blood concentration of total phenols increased rapidly in nephrectomized animals which had the entire intestinal tract simultaneously removed the blood concentration of total phenols either did not increase or increased only slightly.

It is generally thought that the phenolic compounds are produced in the intestinal tract by bacterial decomposition of ingested proteins. The specific bacteria which are responsible for the decomposition of proteins, however, are not known.

The possibility has been investigated that as a result of decreasing the bacterial population of the intestinal tract by the oral use of phthalylsulfathiazole or streptomycin the production and therefore the retention of phenols might be diminished in nephrectomized animals. From the data obtained, the importance of the retention of phenolic compounds in uremia has been evaluated.

#### METHODS

The animals used were healthy, adult mongrel dogs weighing 6 to 14 kilograms and they were maintained before and during the experiment on a balanced diet (Ballard and Ballard). Daily control blood phenol determinations were done usually for two days on thirteen normal dogs. Four of these dogs then received orally a total of 0.5 Gm per kilogram per day of phthalylsulfathiazole in divided doses for three days prior to bilateral nephrectomy and postoperatively until death. To another four animals a total of 0.5 Gm per day of streptomycin was given orally in capsules in divided doses for three days prior to the operative procedure and postoperatively until death. Five control dogs which received no medication were similarly nephrectomized. The operative procedure was a one stage bilateral nephrectomy under ether anesthesia with preanesthetic morphine and atropine. The adrenals were left intact.

Preoperative and postoperative phenol levels were done on each of the animals according to the method of Bernhart and Schneider.<sup>4</sup> Fresh stool specimens from each animal were studied frequently by serial dilutions of 1 Gm wet weight of feces in Bacto Lactosef

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The streptomycin was supplied by Dr D. F. Robertson of Merck and Company Inc Rahway N. J.

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broth before and during the period of drug administration to determine the coliform organism content. The greatest dilution of stool at which both acid and gas were produced in the broth was considered a measure of the number of coliform organisms in the fecal specimens. In the greater dilutions one is probably dealing with coliform organisms, but in the smaller dilutions any aerobic organisms producing gas from proteins or lactose may be included in the measurement.

All the animals were observed frequently until death, and the presence or absence of the various depressive and excitatory symptoms of uremia was recorded. The length of survival after operation was noted in all animals, and each animal was autopsied as soon after death as possible.

## RESULTS

A preliminary study of the blood phenol concentrations in seventy-three determinations on twenty-four normal dogs yielded a mean value of 150 mg per 100 cc, with a range of from 0.82 to 2.30 milligrams. Only two of the

TABLE I. EFFECT OF ORAL PHTHALYSULFATHIAZOLE AND STREPTOMYCIN ON B

DOG (WEIGHT IN KG.)	1		2		3		4		5	
	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT (+)	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT	BLOOD PHENOLS (MG. %)	FECAL COLIFORM COUNT
1 (8.7)	1.67	8	1.37	7	1.54	—	1.78	—	1.69	—
2 (9.5)	2.04	6	1.88	—	2.19	6	1.85	6	1.78	—
3 (10.7)	1.72	6	1.45	—	1.72	7	1.85	—	1.69	—
4 (7.1)	1.37	6	1.17	—	1.20	7	1.05	7	1.00	—
5 (9.5)	1.95	7	1.93	—	1.49	—	1.54	6	1.71	—
<i>Phthalysulfathio</i>										
6 (10.2)	2.02	5	1.88	5	1.70	—	1.84	—	2.10	—
7 (6.4)	2.07	7	2.05	—	1.78	—	1.63	2	1.34	—
8 (11.1)	1.56	7	1.37	—	1.15	—	1.10	2	1.30	—
9 (7.9)	1.10	7	1.36	—	1.52	—	1.11	2	1.74	—
<i>Streptor</i>										
10 (5.7)	1.43	6	1.35	—	1.48	—	—	<2	1.65	—
11 (6)	2.30	6	2.31	—	1.84	—	—	<2	1.01	—
12 (7)	1.25	7	1.59	—	1.55	—	—	<2	—	—
13 (10.7)	—	—	—	—	1.77	—	—	<2	1.60	—

a Lethargic q quiet b labored breathing r retching v vomiting i irregular heart rate  
P Autopsy showed death may have been due to pneumonia.  
F The approximate log of the number of organisms in 1 Gm wet weight of feces obtained by w  
\* Day treatment was started  
† Died seventh day after nephrectomy  
‡ Sulfathiazole blood level free 4.2 combined 0.6 On fifth day blood sulfathiazole level was 2.9 free, 0.0 c  
§ Sulfathiazole blood level free 1.1 combined 0.5 total 4.8 mg per cent  
total 1.6 mg per cent

twenty four dogs had concentrations over 2.0 mg per 100 cubic centimeters. This corresponds closely to normal blood phenol concentrations reported for human beings.

The results in the thirteen experimental dogs are presented in Table I. In five control dogs the mean length of life after bilateral nephrectomy was ninety nine hours ranging from eighty four to one hundred twenty six hours. The four animals which received phthalylsulfathiazole lived from forty five to one hundred sixty nine hours after operation with a mean of one hundred ten hours. The animals which received oral streptomycin lived only from fifty five to eighty one hours postoperatively with a mean survival of sixty eight hours.

BLOOD CONCENTRATIONS AND FECAL COLIFORM ORGANISM COUNTS IN NEPHRECTOMIZED DOGS

6			7			8			9			10		
NEPHRECTOMY														
BLOOD PHENOLS (MG %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG %)	FECAL COLIFORM COUNT	SYMPTOMS	BLOOD PHENOLS (MG %)	FECAL COLIFORM COUNT	SYMPTOMS
<i>Nephrectomized</i>														
3.41	6	—	3.45	6	a	3.68	—	a b, s	4.36	—	a b r v	4.10	Died	a b s v
2.70	—	q	2.85	—	q	3.76	—	a r		Died		—	—	—
2.07	—	q	3.08	—	a	3.96	—	a b v r	4.57	6	a b	—	—	—
1.78	—	q	Died		P	—	—	—	—		—	—	—	—
2.45	—	q	4.63	—	a	5.40	6	a	6.60		a b r	—	—	—
<i>ated Nephrectomized</i>														
3.11	—	a, x	3.65	2	a	4.99	—	a b i g	5.18	—	a b t r	7.31	Died†	a b t v
2.09	2	—	3.11	—	—	3.38	—	a s	3.70	Died	a s t r v	—	—	—
2.20	—	a	4.83	—	a	—	—	—	—	—	—	—	—	—
			Died		§	—	—	—	—	—	—	—	—	—
2.23	—	a	4.60	—	a	5.14	3	a	6.0	—	a s	†	—	—
<i>ated Nephrectomized</i>														
1.30	—	q	1.54	Died	a	—	—	—	—	—	—	—	—	—
1.54	—	—	1.48	2	a	2.09	—	Died	—	—	—	—	—	—
1.60	—	—	2.23	2	a	—	—	Died	—	—	—	—	—	—
1.96	—	—	2.14	<2	a	2.14	—	Died	a r	—	—	—	—	—

very stools & staggering x, abnormally belligerent (snapping growling) t, myoclonic twitching  
 rect of serial dilutions on gas and acid production in lactose broth.  
 died 9 mg per cent total

The blood phenol concentrations of the control animals taken the day of or the day prior to death were from 1.78 to 6.60 mg per 100 cc, with a mean of 4.16 milligrams. The animals receiving phthalylsulfathiazole had blood phenol concentrations at death of from 3.70 to 7.35 mg per 100 cc, with a mean of 5.54 milligrams. The animals given streptomycin died with blood phenol concentrations ranging from 1.54 to 2.23 mg per 100 cc, with a mean of 2.00 milligrams. Drug administration caused no significant change in the blood phenol concentrations in unoperated animals.

No animals showed evidence of predominantly increased nervous system irritability, although occasional coarse muscular twitchings were seen. All showed progressive nervous system depression terminating in death. Almost all of the animals showed severe anorexia, and they retched and vomited during the last forty-eight hours of life, one control animal had diarrhea. There was no significant difference in the uremic signs in the three groups. Some which received streptomycin showed earlier appearance of uremic signs than did the animals in the other two groups. The depth of depression was not always directly proportional to the concentration of blood phenols in the control and phthalylsulfathiazole groups.

Stool bacteriologic examinations revealed that within forty-eight hours of the start of oral phthalylsulfathiazole or streptomycin administration the coliform counts decreased from  $10^6$  to  $10^7$  organisms per gram of wet stool to  $10^2$  or less and this level was maintained until the death of the animal. In general streptomycin reduced the coliform counts somewhat more than did the sulfonamide.

Autopsy examinations revealed nothing of important significance.

#### DISCUSSION

*Relationship Between Blood Phenol Concentration and Uremic Depression*—Becher<sup>1</sup> and Harrison and Mason<sup>2</sup> demonstrated experimentally that signs of depression in uremia are accompanied by an elevation of the blood phenol concentration. This was confirmed clinically by Dickes<sup>1</sup> and Roen,<sup>13</sup> among others, who reported that the correlation between elevated blood phenol concentration and the signs of narcosis was much closer than between the same signs and any other known chemical abnormality in uremia. They therefore concluded that there was an etiologic relationship between the two. This argument was strengthened by the fact that the clinical picture of phenol intoxication resembles the depressive aspects of uremia. On the other hand Nesbit, Burke, and Olsen<sup>7</sup> in studying patients with uremia due to postrenal obstruction concluded that although the blood phenol concentration is elevated in uremia, uremic symptoms, including those of narcosis, are not always related to an elevated phenol concentration. They found no parallelism between the concentration of blood phenols and the intensity of symptoms and signs.

In our animals the administration of streptomycin effectively maintained the blood phenol concentrations at or near normal figures without altering the symptomatology. There appeared to be an earlier onset of symptoms and

earlier death in the streptomycin group. In addition although all control animals and those receiving phthalylsulfathiazole had increased blood phenol concentrations, there was no striking correlation between the severity of narcosis and the concentration of blood phenol. In several instances concentrations over 35 mg per 100 cc were attended by mild depression only while in others blood phenol concentrations lower than this were associated with severe stupor. These findings make it unlikely that blood phenols are etiologically related to the symptoms of depression in uremia. There must be some other retained urinary constituents which produce these symptoms.

The survival times in the control and phthalylsulfathiazole groups agree with those previously reported. Rodbard<sup>6</sup> reported a survival after bilateral nephrectomy ranging from sixty to one hundred thirty hours with a mean of eighty five hours. Harrison and Mason reported a survival in dogs of from eighty five to one hundred twenty hours after the animals became completely anuric. The survival time of the group which received streptomycin was considerably less than that of the other two groups but because of the small number of animals in each group there is some doubt that this difference is significant. The explanation for this is not known since it is known that streptomycin is not absorbed from the intestinal tract in appreciable amounts. However it is possible that degradation or conversion products of streptomycin which would not be measured with the usual methods if they were not bacteriostatic, may accumulate and account for the earlier death.

Since the animals receiving streptomycin died earlier than those in the other two groups it might be argued that the lower phenol concentrations in the streptomycin group could be attributed to this fact. However the mean blood phenol concentration of the control group forty eight hours post operatively was 3.50 mg per 100 cc and the mean forty eight hour blood phenol concentration of the phthalylsulfathiazole group was 4.05 mg per 100 cubic centimeters. At a corresponding time the mean blood phenol concentration of the animals receiving streptomycin was 1.85 mg per 100 cubic centimeters. Streptomycin therefore must effect the intestinal organisms responsible for the major portion of phenol production.

*Phenol Production by Bacterial Action and the Effect of Chemotherapeutic Agents*—Phthalylsulfathiazole<sup>8</sup> and streptomycin<sup>9, 10</sup> taken orally are poorly absorbed from the intestinal tract and thus they exert nearly all of their antibacterial effect within the lumen of the intestine.

Poth and Ross<sup>8</sup> reported that phthalylsulfathiazole markedly depressed coliform and vegetative clostridial counts in human feces but it had no effect on spores or on the *Streptococcus faecalis*. On the other hand Miller<sup>11</sup> has shown in the rat that although coliform organisms are decreased by this drug there is no significant change in the number of aerobic or total anaerobic organisms or in the number of spores in the feces. Segel, Schweinburg and Fine<sup>12</sup> reported that this drug decreases gas formation in obstructed intestinal loops in rats by inhibiting the action of the coli aerogenes proteus group and certain of the clostridia. In our animals the coliform group was markedly

decreased in number by phthalylsulfathiazole administration, we did not study its effect on other organisms. However this drug produced no change in the course of the uremia nor did it diminish the usual increase in the blood phenol concentration. Apparently phthalylsulfathiazole does not act on those organisms in the intestine which are responsible for the production of phenols. Our results agree with those of Banker and Schmidt<sup>12</sup> who reported that the administration of phthalylsulfathiazole to normal dogs resulted in no change in the concentration of phenols in the urine in spite of the almost complete inhibition of fecal coliform organisms. They concluded that the phenol producing organisms were not affected.

Oral streptomycin also markedly diminishes fecal coliform organisms.<sup>14</sup> Zintl<sup>15</sup> gave 1 Gm of streptomycin per day orally in divided doses to fifteen patients and noted a marked depression of fecal coliform and streptococcal organisms and a moderate diminution of clostridia. In comparison, succinylsulfathiazole, a drug similar in its action to phthalylsulfathiazole, diminished fecal coliform organisms moderately and clostridia and *St. faecalis* not at all. Smith and Robinson,<sup>16</sup> using an oral dose of 30,000 to 300,000 units per kilogram in mice, found that streptomycin eliminated all gram-negative organisms and most gram-positive ones from the feces, leaving only a small number of gram-positive spore-forming organisms. We found a marked inhibition of coliform organisms by oral streptomycin, but we did not study its effect on other fecal organisms.

The specific identity of the organisms responsible for phenol production is not known. In considering the possibilities, it appears that the organisms probably would belong to that group manifesting great hydrolytic activity toward proteins, and that they also would be sulfonamide resistant and usually streptomycin susceptible. Of the organisms usually found in feces, the ones with the greatest hydrolytic activity toward proteins<sup>17</sup> are *Clostridium sporogenes* and *Proteus vulgaris*. Of these two, the former is sulfonamide and streptomycin resistant while the latter is sulfonamide resistant but is usually sensitive to streptomycin. There is some basis then for the hypothesis that a small portion of phenol production may be attributed to *Cl. sporogenes* and that *P. vulgaris* is principally responsible for phenol production. It is also possible that the production of phenols requires the integration of the metabolic activities of more than one organism in the production of one or more precursors of phenols.

#### SUMMARY

Blood phenol determinations were done on three groups of dogs. After a control period, usually two days, phthalylsulfathiazole was administered orally to one group of four dogs in divided doses totalling 0.5 Gm per kilogram per day until death. In the same way streptomycin was administered orally to one group of four dogs in divided doses totalling 0.5 Gm per day until death. The third group of five animals served as a control. A one-stage bilateral nephrectomy was done on the experimental groups three days after the drug administration was started and five days after the start of the experiment in

the control group. Fresh stool specimens from each animal were studied frequently by serial dilutions in Bacto Lactose broth to determine the coliform organism content.

The control mean blood phenol concentration was 1.50 mg per 100 cc, with a range of 0.82 to 2.30 milligrams. The control group died with a mean blood phenol concentration of 4.16 mg per 100 cc with a range of 1.78 to 6.60 milligrams. The phthalylsulfathiazole group died with a mean phenol concentration of 5.54 mg per 100 cc with a range of 3.70 to 7.35 milligrams. The streptomycin group died with a mean phenol concentration of 2.00 mg per 100 cc with a range of 1.54 to 2.23 milligrams.

None of the animals showed evidence of predominantly increased nervous system irritability, although occasional coarse muscular twitchings were seen. All animals showed progressive nervous system depression terminating in death. There was no striking correlation between the blood phenol concentrations and the depressive signs of uremia.

It is concluded that the retention of phenolic compounds is of little or no significance in producing the signs of nervous system depression seen in uremia.

Within forty-eight hours of the start of phthalylsulfathiazole and streptomycin administration the fecal coliform counts decreased from  $10^6$  to  $10^2$  or less and this level was maintained until the animals' deaths.

It is concluded that the predominant organism responsible for phenol production in the intestinal tract does not belong to the coliform group and that it is not inhibited by phthalylsulfathiazole but is inhibited by streptomycin. It is suggested that this organism is probably *P. vulgaris*.

The authors are indebted to Dr. Manson Meuls and Dr. MacDonald Fulton for advice on bacteriologic matters and to the staff of the Clinical Chemistry Laboratory for the blood sulfathiazole determinations.

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# MECHANISMS OF DESOXYCORTICOSTERONE ACTION

## I RELATION OF FLUID INTAKE TO BLOOD PRESSURE

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ESSENTIAL hypertension is characterized by a type of sustained blood pressure elevation in which the usual mechanisms for counteracting rises in pressure do not appear to have been brought into operation.<sup>1</sup> Preliminary results of investigations now in progress suggested that desoxycorticosterone may possess an ability, perhaps to a unique degree, to elevate mean pressure without production of secondary cardiac slowing or pulse pressure increase. Consequently it seemed desirable to study the mechanisms by which this drug affects the height of the blood pressure level.

The relatively rapid return to normal pressure levels following adequate desoxycorticosterone acetate (DCA) therapy in Addisonian crisis is usually considered to reflect the restoration of an adequate blood volume consequent upon correction of renal sodium and water loss. On the other hand the hypertension which may develop subsequent to DCA overdosage both in patients with Addison's disease<sup>3</sup> and in normal subjects<sup>4</sup> is not explicable on the basis of abnormal sodium retention<sup>4</sup> or elevated plasma volume<sup>4, 6</sup> and with few exceptions<sup>7</sup> requires a period of weeks to months for its induction.<sup>2, 4, 8</sup> These considerations coupled with the absence of any conclusive demonstration of a contractile action on vascular musculature suggest that DCA induced hypertension may constitute a reaction to some more direct effect of the compound which precedes the pressure elevation in time.

Knowledge of the more immediate action of DCA is concerned largely with its influence on water and electrolyte balance. Although these effects are considered most frequently in relation to tubular reabsorption of sodium their clinical expression is perhaps more evident in terms of over all fluid exchange. Cortical insufficiency is accompanied by a diminished capacity for water excretion<sup>9</sup> while excessive DCA administration is followed by a diabetes insipidus like syndrome.<sup>10</sup> Since it appeared possible that the development of elevated blood pressure might be indirectly related to the profound influence of the salt retaining steroids on fluid exchange it was decided to study the temporal course of the two phenomena in a quantitative manner.

### EXPERIMENTAL PROCEDURES AND RESULTS

The experimental animals for the first group of studies consisted of twenty rats of the Sprague Dawley strain, weighing approximately 65 grams. All animals were kept in separate cages and were fed on Purina laboratory chow. Sodium chloride solution 0.86 per cent, was substituted for drinking water. Consumption of food and fluid was unrestricted. Fluid intake was measured daily and weight weekly. The mean daily fluid intake per gram of body weight

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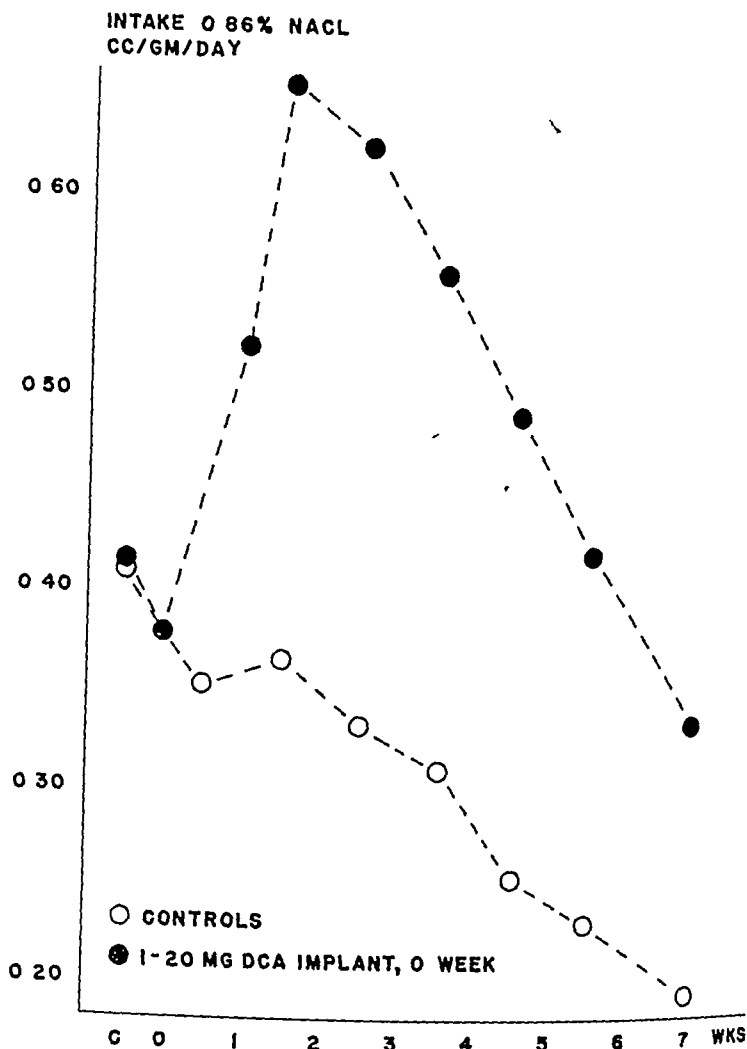


Fig 1—Alterations in fluid intake following DCA implantation in young rat

was calculated for each animal once a week by dividing the average daily intake by the mean weight for the week

At the end of the first week the animals were paired as closely as possible according to weight, unit fluid intake, and sex. Single 20 mg DCA pellets with an absorption time of four to six months were implanted beneath the skin of the back of each of the ten animals comprising the test group, using ether anesthesia. The animals of the control group were sham operated. Measurements of weight and intake were continued as described for a total of eight weeks.

The daily fluid intake of the control group per gram of body weight declined with age in linear fashion as illustrated in Fig 1. The test group, following implantation, manifested an abrupt and marked increase in intake, maximal within ten days, to a value twice that of the control group.

When this maximum increase had been attained, the unit fluid intake thereafter decreased with age in the test group also. However, the rate of de-

eline exceeded that displayed by the control animals so that with the passage of time the unit intake of the test group approached that of the control group despite the marked rise which had followed DCA implantation.

Studies of the relation of these changes to blood pressure and adenal function were carried out on a second test group of thirty rats. In addition to weight and intake determinations as previously described the blood pressure of each animal was measured weekly by an adaptation of the tail method<sup>11</sup>. In order to secure the greatest degree of reproducibility it was found desirable to preheat the animals for twenty minutes at approximately 40° C and to make a series of ten readings at each determination the first five being discarded. Under these circumstances a group of eighteen control animals followed over a three month period manifested blood pressure levels which never exceeded 135 mm Hg.

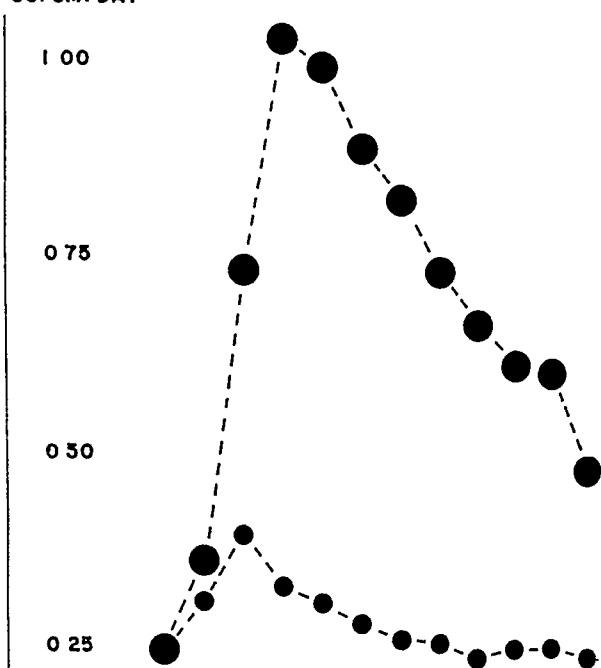
Following a two week control period the test animals were divided into two groups. The twelve rats in the first group were each implanted subcutaneously with ten 20 mg DCA pellets. Six of the animals in this group were adrenalectomized the remainder were sham operated. The eighteen rats comprising the second group were implanted with single 20 mg DCA pellets. Adrenalectomies were performed on twelve animals and sham operations on the remaining six. The sexes were represented equally in each subgroup. Measurements of weight, fluid exchange, and blood pressure were continued as described for a total of twelve weeks.

*Influence of Dosage*—Following implantation all animals manifested the abrupt rise and secondary recession in intake previously described. However the maximum increase in intake was approximately three times as great at the higher dosage level. At the maximal point which was reached in approximately ten days the animals implanted with ten pellets were drinking more than their body weight of saline daily. They appeared to spend the greater part of the time lying on their backs the front paws clasped about the drinking tube from which they would drink rapidly and almost without interruption for minutes on end. During the period which comprised roughly the second week following implantation the animals appeared nervous and irritable and displayed muscular weakness and bouts of transitory paralysis during which the hind limbs were dragged. These phenomena resembled certain of the toxic manifestations which occur in man<sup>2</sup> and in the dog<sup>12</sup> consequent upon depression of serum potassium levels.

When the point of maximum intake had been passed and the period of recession toward lower levels of fluid exchange had set in the general condition of the animals spontaneously improved. They became tractable to handling once more and all evidences of muscular weakness disappeared.

At both dosage levels the blood pressure rose slowly and in reasonably linear fashion throughout the period of observation. However the slope of the pressure curve at the higher dosage was approximately twice as great as that at the lower level. The pressure of the animals implanted with ten pellets averaged 200 mm Hg at the end of twelve weeks as compared with a mean of 160 mm in those bearing a single pellet.

INTAKE 0.86% NaCl  
GC/GM/DAY



BP  
MM HG

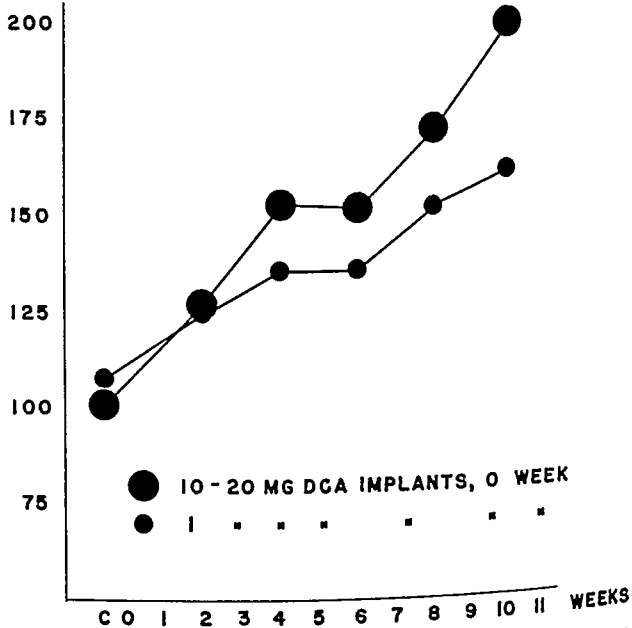


Fig. 2—Relation of fluid intake and blood pressure following DCA implantation in young rats at two dosage levels

When the temporal relation of intake to blood pressure was studied, as illustrated in Fig. 2, it became evident that the earliest effect at both dosage levels was the change in fluid intake which had reached its crest and was declining before the blood pressure became elevated significantly above normal.

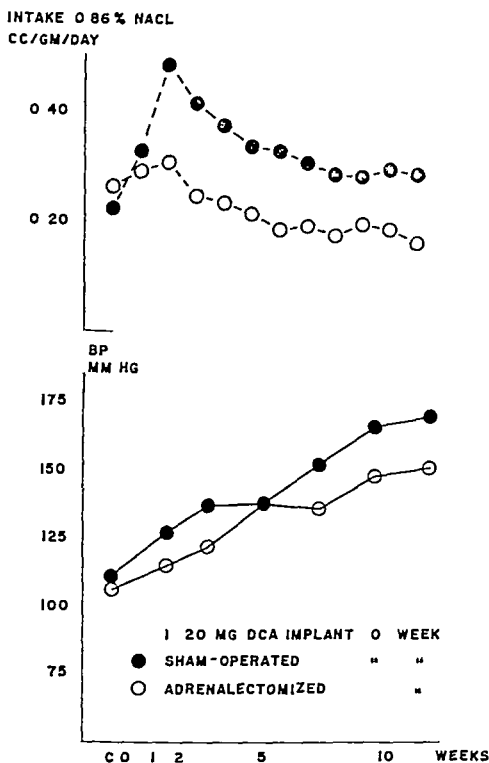


Fig. 3.—Comparison of the responses of adrenalectomized and sham-operated rats to DCA implantation at low dosage.

limits. It also appeared that the intensity of hypertension which subsequently developed was proportional to the maximal increase in fluid exchange while its rate of development was reciprocally related to the secondary regression in intake.

*Influence of Adrenalectomy*—The effect of adrenal removal on DCA action was studied because of observations that adrenalectomized dogs<sup>13</sup> and patients with Addison's disease<sup>14</sup> seemed more susceptible to overdosage than did normal

INTAKE 0.86% NaCl  
CC/GM/DAY

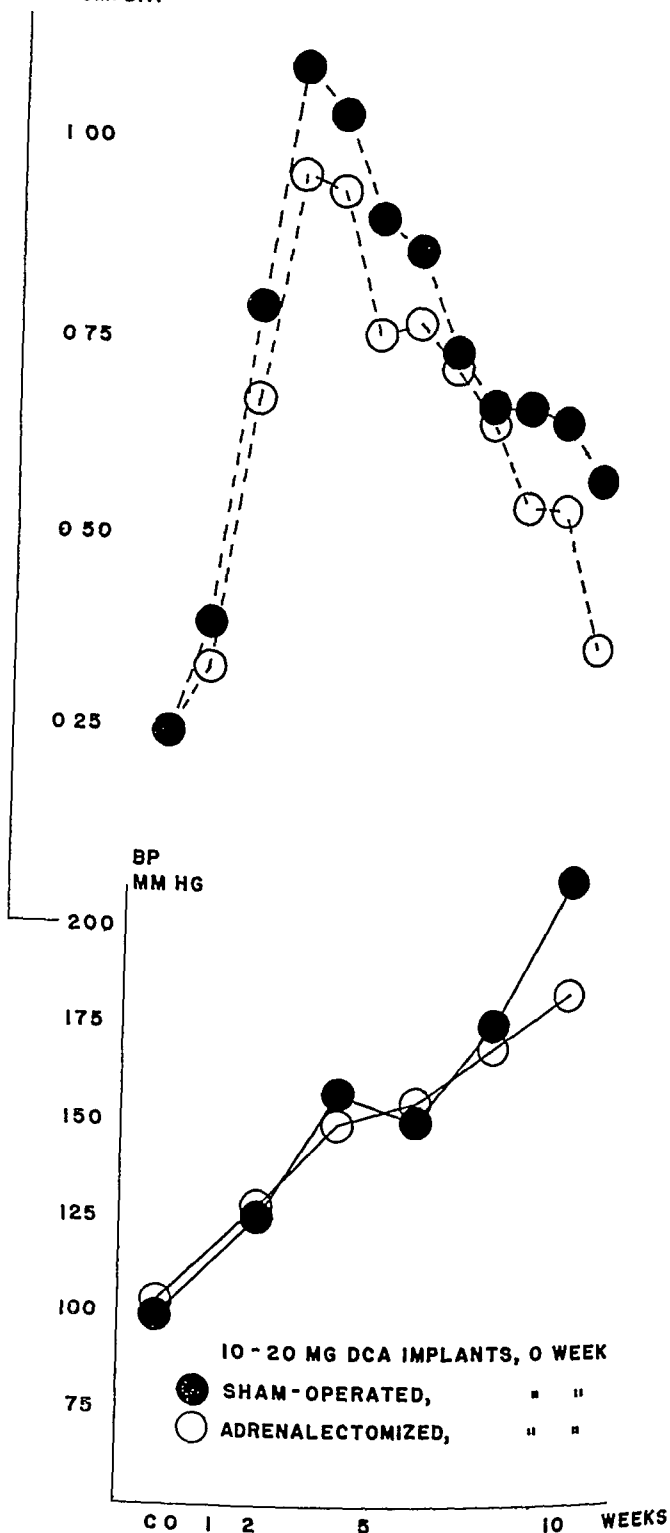


Fig 4—Comparison of the responses of adrenalectomized and sham operated rats to DCA implantation at high dosage

subjects. This apparent hypersusceptibility has been attributed to a lack of antagonistic substances ordinarily secreted by the adrenal.<sup>14</sup>

The implantation of single 20 mg. pellets into adrenalectomized rats was followed by a smaller rise in fluid intake and a lower grade of hypertension than that produced in sham operated animals as illustrated in Fig. 3. These differences became less evident with an increase in dosage as shown in Fig. 4.

#### SUMMARY AND CONCLUSIONS

The immediate effect of DCA implantation in young rats maintained on isotonic saline solution was a rise in fluid intake. The more delayed responses to the drug included a secondary regression of intake values toward control levels and the reciprocal development of hypertension.

The degree of hypertension which developed appeared proportional to the dosage of the drug, the maximal rise in fluid intake and the subsequent rate of decline in intake.

No evidence was found that adrenalectomy sensitized the test animals to the actions of desoxycorticosterone.

The possibility is suggested that the hypertension induced by DCA over dosage may not represent a direct action but may be a compensatory mechanism for overcoming distortions in fluid and electrolyte balance produced by the drug.

We are indebted to Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J., both for generosity in supplying desoxycorticosterone and for many helpful suggestions and criticisms throughout these studies.

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## ACTIVITY OF MICROBIAL ANIMAL PROTEIN FACTOR CONCENTRATES IN PERNICIOUS ANEMIA

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THE presence of an unidentified Factor X in concentrated antipernicious anemia liver extracts was shown in experiments with rats which received a purified diet containing hot-alcohol-extracted casein<sup>1</sup>. Attention has been drawn<sup>2</sup> to the similarity of Factor X to the cow manure factor<sup>3, 4</sup> which promotes growth in chicks and which in turn appears to be similar to the animal protein factor (APF) needed for hatchability of hens' eggs<sup>5</sup> and for chick growth<sup>6</sup>.

In the present investigation it was found that a nonmotile, rod shaped organism from hen feces, when grown aerobically on simplified media containing no appreciable quantities of animal protein factor, could produce this factor,<sup>7</sup> as indicated by assay with chicks on diets containing all the known B-complex factors together with high levels of soybean meal or of alcohol extracted casein. Concentrates were prepared from the growth medium, and by means of the chick assay their potency was standardized against refined liver extract, 10 USP units per milliliter, which produced a similar growth response in chicks. Concentrate I appeared to have between 50 and 100 per cent of the activity of the 10-unit liver extract when injected into chicks. It was prepared by preliminary clarification followed by precipitation with ammonium sulfate. It contained only approximately 0.5  $\mu$ g of tolic acid per milliliter, as indicated by *Streptococcus faecalis* R assay, and the value was not increased by treatment with a chicken pancreas "conjugase" preparation. Concentrate I was administered parenterally to a patient with pernicious anemia.

E. G., a 90 year old white woman, entered the hospital with an erythrocyte count of 1,290,000 per cmm, hemoglobin, 43 Gm per 100 ml, leucocyte count, 3,050 per cmm, hematocrit, 13 per cent, mean corpuscular volume, 100.5 c  $\mu$ , mean corpuscular hemoglobin 33.3  $\mu$ g, mean corpuscular hemoglobin concentration, 33 per cent. The differential blood count, in per cent, was: neutrophils, 75, eosinophiles, 10, lymphocytes, 20.5, monocytes, 3.0. The neutrophils had highly segmented nuclei and were large cells. The erythrocytes showed marked variation in size and shape and appeared macrocytic. The platelets were decreased in number.

Gastric analysis showed absence of free hydrochloric acid after stimulation with histamine. Stereal marrow aspiration revealed a marrow with increased cellularity, a relative and absolute increase in nucleated red cells, and a megakaryoblast content of 24.4 per cent.

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\*The isolation of the active organism and the microbiologic production of animal protein factor concentrates were under the direction of Dr. Milton A. Petty, Lederle Laboratories Division, American Cyanamid Company. These studies will be the subject of a separate publication at a later date.



TABLE I PATIENT E. G. GIVEN APF CONCENTRATE I, CITRATED WHOLE BLOOD (500 ML) ADMINISTERED ON SECOND DAY OF TREATMENT

DAY OF TREAT- MENT	RBC (MIL- LIONS PER CMM)	HB (GM PER 100 ML)	WBC (PER CMM)	PLTICL COUNTS (%)	HE- MATO- CRIT (%)	MCV (Cμ)	MCH (μmc)	MCHC (%)	INITIAL MUSCULAR THERAPY
									APF con- centrate I (ml)
0	129	43	5050	12	15	101	3	33	10
1	107	37	4550	08					10
2	099	35	6550	08					10
3	186	57	4700	08					10
4	163	50	3850	16					10
5	195	60	4550	27					10
6	190	63	5900	41					10
7	184	63	4450	68					10
8	141	51	8050	112					10
9	198	65	12400	180					10
10	188	60	6900	206					-
11	194	72	-----	178					-
12	190	61	6100	151					-
13	238	75	7300	126					
14	239	78	7300	101					
15	201	69	4500	69					Purified liver ex- tract
16	243	77	6100	51					10 u ml
17	237	79	5450	48	23	97	1	4	(ml)
18				12					10
19	193	82	6900	22					10
20	210	81	7850	24					10
21	212	7	5800	21					10
22	237	82	5000	32					10
23	229	78	4500	42					10
24	238	76	3000	60					10
25				47					10
26	258	86	4000	45					10
27	290	89	4050	45					
28	249	89	3900	35					
29	273	92	4450	25					-
30	298	92	3800	18	20	101	31	31	-
31	200	93	5250	19					30

Lederle

Animal protein factor concentrate I was given in daily intramuscular dose of 10 ml (Table I) for nine doses. The patient was very ill with severe nausea and fecal vomiting. X-ray studies of the abdomen showed multiple distended loops of small intestine. A Miller Abbott tube was inserted, but after the third dose of animal protein factor concentrate the nausea and vomiting ceased and did not reappear. A single blood transfusion of citrated blood (500 ml) was administered when the erythrocyte count fell to 990 000 per cmm on the third hospital day. The patient made a rapid symptomatic recovery, becoming more alert and active and appetite returned. Thereafter the clinical course was uneventful.

A peak of 206 per cent reticulocytes occurred on the tenth day after the start of treatment with animal protein factor concentrate. This was followed by a prompt increase in the numbers of erythrocytes, leucocytes and platelets and in the hemoglobin level (Table I). The reticulocyte response was more delayed than the average response resulting from effective intramuscular therapy with liver extract, but the level reached by the reticulocytes was nearly that of the theoretic maximum.

A course of liver extract was started eighteen days after the beginning of therapy with animal protein factor concentrate. The patient received nine daily intramuscular doses

of 10 ml of purified liver extract (Lederle), each 10 ml containing 10 antipernicious anemia units. On the sixth day of treatment with liver extract there was a second, small reticulocyte peak of 6.0 per cent. The patient continued to improve.

Animal protein factor concentrate II was prepared by clarification with out precipitation with ammonium sulfate. It had between 25 and 40 per cent of the activity of 10-unit liver extract when fed to or injected into chicks. It contained about 0.02  $\mu\text{g}$  of folic acid per milliliter, as indicated by assay with *St. faecalis* R, and the value was not increased by treatment with "conjugase." The concentrate also was assayed for total pteroylglutamic acid with chicks and was found to contain not more than 5  $\mu\text{g}$  per milliliter. In another hospital this concentrate was administered parenterally to a patient with pernicious anemia, and the findings were placed at our disposal.

E. N., an 80 year old white woman, entered the hospital with an erythrocyte count of 1,890,000 per cmm, hemoglobin, 7.7 Gm per 100 ml, leucocyte count, 2,400 per cmm,

TABLE II PATIENT E. N. GIVEN APF CONCENTRATE II

DAY OF TREATMENT	RBC (MILLIONS PER CMM)	HB (GM PER 100 ML)	WBC (PER CMM)	RETICULO CYTES (%)	HE MATO CRIT (%)	MCV (C $\mu$ )	MCH ( $\mu\text{g}$ )	MCHC (%)	INTRAMUSCULAR THERAPY APF concentrate II (ml)
-4	1.89	7.7	2,400	0.4	24	127	41	33	
-2	1.80	7.7	3,700	1.0	23	129	43	33	
-1				0.4					
0	1.87	7.8	2,700	0.9	24	128	42	33	10
1				2.4					10
2	1.98	8.2	3,900	1.9	26	133	41	31	10
3				1.9					10
4	2.08	7.9	6,000	3.7	24	116	38	33	10
5				6.2					10
6	2.17	8.1	4,400	6.4	26	120	37	32	10
7				9.7					10
8	2.28	8.2	----	6.1	26	113	36	32	10
9				6.0					10
10	2.63	9.0	5,600	3.5	30	114	30	30	10
11				1.8					10
12									10
13	3.0	9.5	6,900		31	105	32	30	10
14									10
15									10
16									10
17	2.81	8.9	6,900		31	109	32	29	10
18									10
19	3.18	9.3	7,300		32	99	29	29	10
20									10
21									10
22									10
23	3.33	9.8	6,300		34	100	29	29	10
24									10
25									10
26									10
27									10
28									10
29									10
30	3.38	10.8	11,600	1.0	35	102	31	32	10

hematocrit, 24 per cent mean corpuscular volume 127 c  $\mu$  mean corpuscular hemoglobin 41  $\mu$ g, mean corpuscular hemoglobin concentration 33 per cent

Gastric analysis showed absence of free hydrochloric acid after stimulation with histamine. Sternal marrow aspiration revealed megaloblastic hyperplasia.

The presenting complaint was inability to walk. The patient had a cloudy sensorium. There was loss of vibratory sensation in the lower extremities.

Animal protein factor concentrate II was given in daily intramuscular doses of 10 ml (Table II) for twenty-four days. A reticulocyte peak of 9.7 per cent occurred on the seventh day after the beginning of treatment. There was a prompt increase in the number of erythrocytes and leucocytes and in the hemoglobin level (Table II).

Coincident with the hematologic response there was clinical improvement. The patient became better oriented and there was a return of appetite. The observers considered the clinical improvement to be moderately pronounced.

After thirty days of treatment the erythrocyte count had risen to 3,380,000 per c mm and hemoglobin to 10.8 Gm per 100 ml. The leucocyte count was 11,600 per cubic millimeter. There was a decrease in the mean corpuscular volume and in the mean corpuscular hemoglobin.

#### DISCUSSION

The results indicate that the concentrates of material produced microbiologically, and found to exert animal protein factor activity in chicks, also were active in inducing an hematopoietic response in pernicious anemia.

It is not possible to conclude that the hepatic and bacterial substances responsible for the activity in chicks are identical with the classic antipernicious anemia factor, since the so-called refined liver extracts used as well as the bacterial concentrates, are relatively crude materials. The nearly maximal reticulocyte response to concentrate I occurred on the tenth day while the patient given the weaker concentrate (II) had a submaximal reticulocyte response on the seventh day. With liver extracts of such potency (10 and 25 to 4 units per cubic centimeter respectively) a peak reticulocyte response would have been expected earlier than occurred in the first case. However, the apparent delay in the reticulocyte response may have been due to the very critical state and the advanced age (90 years) of the patient. On the other hand, the delay might reflect a difference in the rate of utilization of the active principles of liver extract and the animal protein factor concentrate.

Whether the second small reticulocyte response that followed the administration of the 10 unit liver extract to patient L. G. indicates that the liver extract was more effective than the animal protein factor concentrate is difficult to determine, since to permit subsidence of reticulocytosis the patient was without therapy for nine days after the course of animal protein factor concentrate was concluded. In any event the animal protein factor concentrate given parenterally produced an almost theoretically maximal reticulocyte response and an increase in the levels of erythrocytes, leucocytes, platelets and hemoglobin, in addition to causing satisfactory clinical improvement.

Conceivably, the bacterial extract contains forms or complexes of the antipernicious anemia factor that are utilized by the chick as sources of animal protein factor activity but are less effectively utilized by the human patient as sources of antipernicious anemia factor activity.

In the second patient the reticulocytes failed to attain a theoretically maximal level, but there, also, there was rapid improvement in the levels of erythrocytes, leucocytes, and hemoglobin, in addition to a satisfactory clinical response.

These findings show that a concentrate prepared from a bacterial filtrate contains a substance capable of producing an hematologic and clinical response in pernicious anemia. Whether the substance is identical with the anti pernicious anemia factor or the recently isolated vitamin B<sub>12</sub>,<sup>9</sup> shown to be active in pernicious anemia cannot be decided at this time.

#### SUMMARY

Concentrates of microbiologically produced material, highly active as a source of the animal protein factor, as measured by assay with chicks, were shown to be effective, when given parenterally, in producing an hematopoietic response in pernicious anemia.

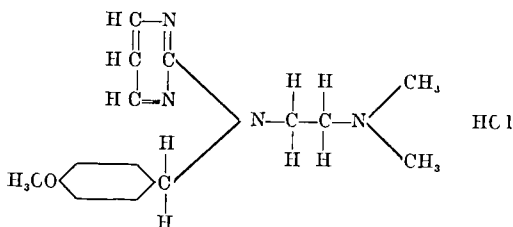
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# EXPERIMENTAL AND CLINICAL STUDIES OF NEOHETRAMINE, A NEW ANTIHISTAMINIC AGENT

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NEOHETRAMINE is one of a new group of synthetic antihistaminic agents found to produce symptomatic benefit in certain allergic states. It is the methoxy derivative of an earlier preparation, Hetramine<sup>1</sup> and is the pyrimidine isostere of another potent antihistaminic drug, Neoantergan<sup>2</sup>



2 (N dimethylaminoethyl N p methoxybenzyl) aminopyrimidine  
monohydrochloride (Neohetramine)

The present report deals with the antihistaminic and antianaphylactic properties of Neohetramine as determined in the intact guinea pig as well as the clinical results observed in a large group of patients with various allergic syndromes.\*

## EXPERIMENTAL

*Antihistaminic Effect in Guinea Pigs (Table I)*—Antihistaminic activity was determined by treating guinea pigs with Neohetramine fifteen to twenty minutes prior to a toxic dose of histamine. 0.4 mg per kilogram of histamine, calculated in terms of the base, was found to be uniformly fatal when injected into the penile veins of untreated guinea pigs. All animals which received 5 mg per kilogram of Neohetramine intraperitoneally prior to this shocking dose of histamine lived, while 50 per cent of those treated with 1.0 mg per kilogram of the drug survived this ordinarily lethal dose of histamine.

*Antianaphylactic Effect in the Guinea Pig (Table II)*—Guinea pigs 400 to 500 grams in weight, were sensitized by the subcutaneous injection of 0.1 cc

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Neohetramine was supplied by the Department of Medical Research, Nepera Chemical Co., Inc., and is now distributed by Wyeth Incorporated, Philadelphia, Pa.

TABLE I PROTECTIVE ACTION OF NEOHETRAMINE IN HISTAMINE SHOCK

NUMBER OF GUINEA PIGS	NEOHETRAMINE (MG/KG I P)	HISTAMINE (MG/KG I V)	PER CENT SURVIVAL
15	None (control)	0.4	0
8	1.0	0.4	50
10	3.0	0.4	40
15	5.0	0.4	100

of horse serum and were given a challenging dose of 0.5 cc in a penicillin twelve days later. Protection against fatal anaphylactic shock was determined by giving Neohetramine intraperitoneally fifteen to twenty minutes prior to the shocking dose of antigen. All untreated controls died in typical anaphylactic shock. Seven out of ten animals which received 3.0 mg per kilogram of Neohetramine lived, while only four out of ten of those pretreated with 1.0 mg per kilogram of the drug survived. All guinea pigs receiving 0.1 mg per kilogram of Neohetramine died.

TABLE II PROTECTIVE ACTION OF NEOHETRAMINE IN ANAPHYLACTIC SHOCK

NUMBER OF GUINEA PIGS	NEOHETRAMINE (MG/KG I P)	PER CENT SURVIVAL
14	None (control)	0
10	0.1	0
10	1.0	40
10	3.0	70

## CLINICAL

*Dosage and Toxicity*—Clinical experience with Neohetramine indicated that symptomatic benefit, if it occurred, would become evident within thirty minutes after ingestion of the drug and last for several hours. Patients usually were instructed to take the drug at four- to six-hour intervals, but in some instances it was found necessary to use the drug more frequently in order to control symptoms. Those with periodic difficulty were advised to take the drug only when symptoms occurred. Initially 50 mg doses were prescribed and later were increased to 100 mg if clinical benefit was not evident. The optimum dose for most adults was found to be 100 mg, while 50 mg doses were employed with good results in children 6 to 12 years of age. Proportionately smaller amounts were used in younger children.

The incidence of side action from Neohetramine was less than that seen with other antihistaminic drugs previously studied.<sup>3</sup> Several patients in this series who previously were found unable to tolerate antihistaminic medication were able to take clinically effective doses of Neohetramine without difficulty. Among 140 patients who were given Neohetramine, only seventeen (12 per cent) complained of side effects. Drowsiness, which is the most frequently encountered untoward reaction from antihistaminic drugs, was noted by five patients, five others reported gastrointestinal irritation, three complained of vertigo, and one patient each experienced weakness, tinnitus, diplopia, and pruritis. In no instance was the side action of a severe degree. Evidence of chronic toxicity as

determined by repeated blood counts and urine examinations was not encountered in those using the drug over longer periods of time. Periodic examinations in four patients who received an average dose of 200 mg daily for six months showed no abnormalities in urine or blood count.

*Symptomatic Effect (Table III)*—Neohetramine was used in 140 patients with one or more of the following allergic complaints: seasonal hay fever, nonseasonal allergic rhinitis, urticaria and angioneurotic edema, asthma, allergic

TABLE III. CLINICAL RESULTS WITH NEOHETRAMINE

CONDITION TREATED	NUMBER OF CASES	HELPED		NOT HELPED	
		NUMBER	PER CENT	NUMBER	PER CENT
Bronchial asthma	40	11	27.50	29	72.50
Vasomotor rhinitis	50	26	52.00	24	48.00
Hay fever	58	37	63.82	21	36.18
Urticaria					
Acute	6	6	100.0	0	0.0
Chronic	4	2	50.0	2	50.0
Dermatitis					
Atopic	3	0	0.0	3	100.0
Contact	2	1	50.0	1	50.0
Unclassified	4	3	75.0	1	25.0
Allergic headache	3	0	0.0	3	100.0
Allergic conjunctivitis	1	0	0.0	1	100.0

dermatitis, headache and conjunctivitis. Thirty of these subjects had two allergic syndromes such as rhinitis and asthma or hay fever and urticaria and since the effect of Neohetramine on each was not necessarily the same in that patient each symptom is listed separately in the accompanying table.

*Seasonal Hay Fever*—A beneficial effect on rhinorrhea, itching and sneezing was seen in 64 per cent of patients with acute hay fever due to pollen or fungus spores. In common with other antihistaminic drugs the relief following each dose was slight in some instances and marked in others but seldom complete. The many factors which influence symptoms in hay fever also affect the response to these drugs. Benefit is usually more evident early in the pollen season and on days when pollen or mold concentration is low. In general patients with mild symptoms, or those with some degree of immunity through desensitization therapy, obtain more relief than those with severe symptoms.

*Allergic Rhinitis Nonseasonal*—Fifty-two per cent of this group obtained some symptomatic benefit from the drug. Rhinorrhea and sneezing were usually more favorably affected than nasal bloating.

*Urticaria and Angioneurotic Edema*—Relatively few of these cases are included in this series. In six patients with acute urticaria marked symptomatic action was apparent following the use of Neohetramine. Two patients with chronic urticaria were consistently relieved by the drug while two others failed to obtain any appreciable help.

*Asthma*—Approximately 27 per cent of asthmatic patients reported some help from the use of the drug. The degree of benefit in these patients in our opinion, was not striking.

*Miscellaneous* The drug was occasionally helpful in alleviating the pruritis associated with allergic dermatoses. Three patients with headache and one with conjunctivitis, all of allergic etiology, were not relieved by the drug.

#### COMMENT

Neohetramine compares favorably with other synthetic antihistaminic drugs previously studied. While antihistaminic and antianaphylactic activities as demonstrated in the intact guinea pig are somewhat less than those determined for several other drugs of this series, its symptomatic action in allergic states is approximately equivalent to other compounds when employed in the optimum dosage of 100 milligrams.<sup>3</sup> A decided advantage which Neohetramine enjoys is the relative freedom from severe side effects accompanying its use. It appears to be especially indicated in those patients who are found to have a low tolerance for other antihistaminic drugs.

Experience with antihistaminic therapy has shown a rather wide individual variation in the clinical response to these compounds. A trial of several drugs in the same patient frequently will reveal one which is particularly effective and well tolerated. The incidence of symptomatic benefit from such medication is therefore increased by the availability of the newer members of this group of synthetic compounds. Our clinical experience would indicate that Neohetramine is a valuable addition to this growing list of antiallergic agents. It must, of course, be remembered that antihistaminic drugs are purely palliative medication and demonstrate no curative action in allergic disease.

#### SUMMARY

Neohetramine, [2-(N-dimethylaminoethyl-N-p-methoxybenzyl) aminopyridine monohydrochloride], prevented fatal shock from intravenous histamine in the intact guinea pig. A similar protective action was demonstrated in guinea pig anaphylaxis.

Oral administration of the drug afforded symptomatic relief to many patients with seasonal and nonseasonal allergic rhinitis, allergic dermatoses, asthma, urticaria and angioneurotic edema.

A relatively low incidence of side effects occurred with the use of Neohetramine. It was found particularly useful in patients unable to tolerate other antihistaminic drugs.

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## TRICHOSTRONGYLUS INFECTION IN HUMAN BEINGS NEED OF DIFFERENTIATION FROM HOOKWORM

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IN 1916 Ransom,<sup>1</sup> in discussing the transmissibility of certain nematodes of ruminants to man stated that *Trichostrongylus* was not an exotic form occurring in remotely distant regions but a parasite which had a direct and immediate interest for American physicians. Though only a few cases of *Trichostrongylus* infection in human beings have been reported in the American literature we believe that this is due to a lack of knowledge regarding the differentiation of these ova from those of hookworm rather than to the rarity of the infection. In casual examinations of feces *Trichostrongylus* ova can be and probably often have been, mistakenly identified as slightly atypical hookworm ova.

Loos<sup>2</sup> (1895) was the first to divide *Trichostrongylus* into four species. Jimbo<sup>3</sup> (1914) undertook a statistical study of the spread of parasitic illness among the Japanese through feces examinations. He found that in Japan where ancylostomiasis is especially prevalent there were many people who had a peculiar type of *Ancylostoma* eggs which because of their great resemblance to *Ancylostoma* had been unjustly identified as such. The specimens appeared different from those described by Loos and therefore were named *Trichostrongylus orientalis*. Jimbo could not state what pathologic importance these worms had, but did recommend the use of oleum chenopodium as a therapeutic measure. Sandground<sup>4</sup> (1936) who had infected himself with *Trichostrongylus* larvae to determine their longevity found that tetrachlorethylene and carbon tetrachloride were ineffective in expelling the worms. There was no diminution in the number of eggs after eight and one half years. In 1938 Schenken and Moss<sup>5</sup> reported the first case of human infection with *T. colubriformis* in the Western hemisphere. They found a single adult worm in a surgically removed appendix. No definite information could be obtained regarding the source of the infection. Maplestone<sup>6</sup> (1941) found a 9 to 25 per cent infection rate in India with a 10 per cent infection rate among 50 Europeans. He considered the recognition of *Trichostrongylus* important. Patients were often referred to as suffering from incurable hookworm infection because they were still passing eggs after several treatments with recognized efficient hookworm drugs. Because of failure to recognize the eggs the patients had received thymol carbon tetrachloride and oil of chenopodium which do have a certain degree of danger. Tsuchiya and Reller (1945) found *Trichostrongylus* ova in a patient who had worked as a farm laborer in the southern states. They thought it conceivable that the infection might have occurred through accidental ingestion of larvae.

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abdominal cramps and nausea. Therapy was discontinued on the sixth day. Prior to this treatment the patient had noted symptomatic improvement with the use of bland diet and Amphogel. At this time she refused to try any further drugs and was continued on the regimen until discharge on May 29, 1947. At the time of discharge there were occasional loose bowel movements, rare ova of *Trichostrongylus* in the feces, a sedimentation rate of 30 mm per hour, two eosinophiles in the differential count, and no evidence of anemia. The sputum had shown few to many colonies of acid fast bacilli during the flare up in September, 1946, but for six months prior to discharge all concentration and cultural examination of sputa and gastric lavages had been negative for acid fast bacilli. The pulmonary disease appeared arrested with pneumothorax.

When the ova had been properly identified, the patient's family were advised to have a checkup. Two brothers failed to show any ova on two occasions. The patient's mother, however, had many *Trichostrongylus* ova in December, 1946, and a few ova and an eosinophil count of seven in January, 1947. The mother reported that she had always been in poor health. As far as she could remember she had never had any abnormal bowel movements. A specimen of the mother's feces was sent to the Army Medical School at Washington, D. C. The report stated that *Trichostrongylus* ova were present, again confirming the diagnosis.

#### COMMENTS

Detection of the ova of *Trichostrongylus* is difficult due to the small number usually present. A concentration and levitation technique may be required (Willis<sup>10</sup>). They are frequently mistaken by casual observers for the ova of hookworm, which they closely resemble. *Trichostrongylus* ova are characterized by a transparent shell membrane which is thicker and more lustrous than that of the hookworm. They are elongated, with the ends more pointed than those of the hookworm, though a great number will show some rounding at one end—like a hen's egg (Maplestone). The ova of hookworms average about 64  $\mu$  in length and 41  $\mu$  in width, while those of *Trichostrongylus* are much longer and slightly wider. Jimbo found the ova to average 83 to 90  $\mu$  in length and 41  $\mu$  in width, Tsuchiya and Reller 81 to 97 by 40 to 53  $\mu$ , with an average of 86 by 43  $\mu$ , O'Neal and Magath 76 to 86 by 44 to 47 microns. The ova of Patient J. L. in this report averaged 91  $\mu$  in length (range 80 to 100) and 44  $\mu$  in width (range 41 to 50), and those of her mother 88  $\mu$  in length (range 80 to 98) and 43  $\mu$  in width (range 40 to 46). The morula stage of *Trichostrongylus* will show at least sixteen divisions, a somewhat later stage of segmentation than that of hookworm ova. The individual divisions are equal in size, almost circular, and of a grapelike appearance. This is an important aid in differentiation. Two larvae were found, measuring approximately 285  $\mu$  in length and up to 24  $\mu$  in width. Jimbo has described the roundworm as being thin, delicate, sexually divided, of colorless to gray-white appearance, ranging from 380 to 670  $\mu$  in length and up to 83  $\mu$  at its widest portion.

According to Craig and Faust<sup>11</sup> several hundred worms are necessary to provoke marked clinical manifestations. Jimbo also notes that the severity of symptoms will depend on the number of parasites. Maplestone, however, states that as far as he knows the worms never give rise to any objectionable symptoms. Two of O'Neal and Magath's patients had intermittent blood tinged stools, while the third had abdominal cramps and frequent loose stools. The daughter (Patient J. L.) in our cases had diarrhea for several years while the mother had no symptoms. Yet the mother on occasions showed showers of ova.

Since so few cases of human *Trichostrongylus* infection have been reported the mode of infection is not definitely known. Chandler<sup>12</sup> believes that the infection occurs through the ingestion of contaminated vegetable matter rather than by penetration through the skin. It is possible that grazing lands are being seeded with eggs, since goats and sheep are natural hosts. Koino<sup>13</sup> in experiments on mice traced the migration of the larvae and they seemed to follow the same route as hookworm larvae. He obtained penetration and migration to the lungs both by the oral route and through the skin. It would seem probable that human beings could acquire *Trichostrongylus* infection in the same manner as they do hookworm.

From all reports it appears impossible to eradicate the *Trichostrongylus* with the usual anthelmintics. As seen from previous investigations and from our own efforts the use of oil of chenopodium, thymol, carbon tetrachloride, tetrachlorethylene, as well as emetine hydrochloride, carbarsone and gentian violet is ineffective. Where *Trichostrongylus* has been mistaken for hookworm the patients have been subjected to needless objectionable and perhaps dangerous treatments.

#### CONCLUSIONS

There are we realize a number of questions left unanswered in this report. We do not know how the infection was acquired. We do not know whether the infection was acquired simultaneously by mother and daughter or whether it was transmitted from one to the other.

We cannot offer any course of therapy for the eradication of *Trichostrongylus*. The usual anthelmintics have been found ineffectual in the so called incurable hookworm infection which may be a *Trichostrongylus* infection.

In areas endemic for hookworm as well as in individual cases the ova of hookworm should be carefully examined and differentiated from those of *Trichostrongylus*. A lack of knowledge regarding the latter led to an original diagnosis of *Necator americanus* in our patient. If remembered in differential diagnosis, however, the shape, larger size and advanced segmentation of the *Trichostrongylus* ova cannot fail to attract the attention of the investigator.

The significance of the infection as related to the future health of patients can only be determined by prolonged follow up and by further case reports.

We wish to thank Dr. H. T. Uehya, Washington University School of Medicine, St. Louis, Mo., and Lt. Col. G. W. Hunter and Major R. Traub of the Army Medical School, Washington, D. C., for checking the identity of the ova, and Mr. M. Dietrich, University of Buffalo Medical School, Buffalo, N. Y., for taking the photographs.

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## LABORATORY METHODS

### ERYTHROCYTES IN URINARY SEDIMENT IDENTIFICATION AND NORMAL LIMITS

WITH A NOTE ON THE NATURE OF GRANULAR CASTS

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ALTHOUGH the importance of erythrocytes in the urine has long been recognized and then significance a subject of much discussion there has been little attempt to confirm their identification. The average laboratory technician fails to note the presence of red blood cells unless their number is definitely above the upper limits of normal.<sup>1</sup> Unfamiliarity with the varied forms of erythrocytes in urinary sediments, cursory examinations and inadequate technique are the main reasons for this failure. Addis noted that care was necessary to avoid passing over fragmented or partially lysed cells which may cast only faint shadows. The importance of the problem is emphasized by a 2 per cent incidence of hematuria in 20 000 consecutive patients in clinic practice<sup>2</sup> and a 10 per cent occurrence of erythrocytes in urinary sediments examined in our laboratory.

Two approaches have been used in this country to aid detection and to cope with the problem of identification (1) the quantitative work of Addis and (2) tests for hemoglobin and related substances utilizing benzidine or orthotolidine. The first technique is tedious and inconvenient for clinical use while in the second the reagents may react with interfering substances and do not morphologically identify erythrocytes. Aware of the confusing variations in size shape and optical density of erythrocytes in the urinary sediment and of the responsibility of even an experienced technician for correct identification we have modified a tissue stain<sup>3</sup> which appears to be specific for red blood cells.

#### Stock solutions

10 per cent aqueous solution of benzidine hydrochloride  
(Merek)

20 per cent aqueous solution of sodium nitroprusside

300 per cent hydrogen peroxide (superiodol)

The benzidine and nitroprusside solutions keep from four to five months in brown bottles if not exposed to direct sunlight.

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## Working solutions

- A 0.1 per cent benzidine in 0.2 per cent nitroprusside  
 1 c c of the 1.0 per cent aqueous solution of benzidine  
 (stock) is diluted to 6 to 7 c c with distilled water  
 1 c c of the 2.0 per cent aqueous solution of sodium nitro  
 prusside (stock) is added and then distilled water to  
 10 c c

This solution is stable for about two weeks, it should be discarded as soon as a precipitate is formed

- B 3.0 per cent hydrogen peroxide  
 1 c c of superoxol is made up to 10 c c with distilled water  
 This solution deteriorates in from three to four days

## TECHNIQUE

A drop of sediment is placed on a slide and a drop of benzidine nitroprusside solution is added. Thorough mixing is accomplished by tilting the slide or by stirring with the cover glass. A drop of dilute peroxide solution is then added and carefully mixed.

Sediments of alkaline specimens, which do not stain readily, may be treated within the tip of the centrifuge tube with 1 or 2 drops of 10 per cent nitric acid to destroy the crystals and lower the pH. In our experience this has not caused hemolysis of red cells, probably because of the fixing property of urine and the presence of basic salts. After three washings with 0.9 per cent saline solution these sediments give results comparable to those attained in acid urines. Urine specimens of a high specific gravity, especially those containing preservatives, may react slowly to the stain, and should stand five to eight minutes before examination. Washing three times with 0.9 per cent saline solution should accelerate staining.

## RESULTS

The erythrocytes are stained a deep blue-purple. This color may be pale, even a light blue if the cell has deteriorated. Some cells will have scattered deep blue punctate granules. Suspensions of yeast cells in normal urine less than twenty-four hours old may show a slight blue-green indescence which is not confused with the deeper color and less refractive appearance of the stained erythrocytes, while those over twenty-four hours old have no affinity for the stain. Leucocytes may take a pale-gray appearance in the cytoplasm with slightly dark and irregular staining of the nucleus. No granules have been observed in the cytoplasm of white blood cells or epithelial cells. Hyaline casts do not take the stain. The granules of fine and coarse granular casts have the deep bluish-purple hue of stained erythrocytes. The red blood cells of casts stain similarly to those free in solution. Fungi and spores show no response to the stain.\* Pollen, diatoms, prostatic bodies, and other elements of urine have no affinity for the stain.

\*We are indebted to Mr. Robert K. Steffa, Waterloo, Ia., for submitting fifteen species of fungi and three strains of yeast.

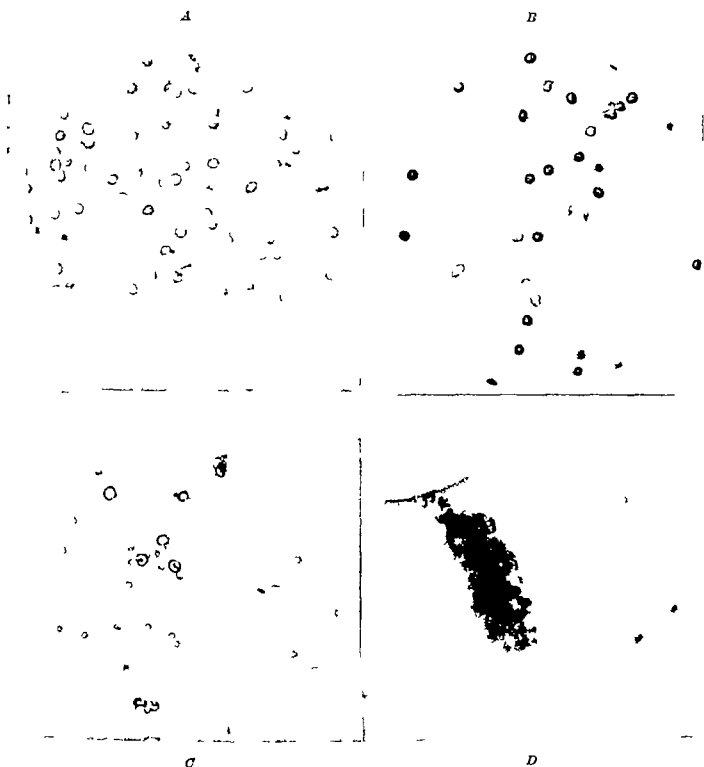


Fig 1—A Red cells unstained B, Same cells stained. Note variable degree of stain intensity C, Suspension of yeast and red cells stained D Composite print. Granular casts (and few red cells) stained and unstained

#### STAINING REACTION

Since this stain seems to have a specificity for red blood cells the probable site of action is hemoglobin or one of its heme derivatives. Benzidine and oxidizing agents such as hydrogen peroxide react with acid solutions of hemoglobin to produce a blue color which according to Wu<sup>5</sup> is exactly proportional to the amount of hemoglobin present. Bing and Baker<sup>6</sup> consider this a pseudoperoxidase reaction. Benzidine and hydrogen peroxide react slowly with intact red cells in the urine to produce after some time a faint blue color. Sodium nitroprusside greatly increases the speed and intensifies the color of the reaction.

The mechanism of this reaction is very complex. It is known however that solutions of benzidine subjected to nascent oxygen or sodium ferrieyanide

rapidly form a deep blue precipitate. Solutions of benzidine and sodium nitroprusside form a similar precipitate only after standing for days. Benzidine, a fundamental dyestuff, is attached to the protein of red cells. Hemoglobin containing ferrous iron ( $\text{Fe}^{++}$ ) is oxidized to methemoglobin containing ferric iron ( $\text{Fe}^{+++}$ ) by hydrogen peroxide.<sup>7</sup> The nitroprusside might react by forming a colored iron-cyanide compound or alter the reaction by its nitro group. Since we obtained no results under the conditions of our stain substituting sodium ferriocyanide, sodium ferrocyanide, or nitrites, it appears that nitroprusside may be a catalyst.

Because of these findings we feel that benzidine may be an indicator of an oxidation-reduction system<sup>8</sup> which results in the oxidation of benzidine and the development of a deep blue color. The nitroprusside may act as a catalyst.

#### DISCUSSION

*Identification*—Most efforts at identifying blood in the urine have been directed toward the development of color tests for hemoglobin and related substances. Benzidine,<sup>9-10</sup> toluidine,<sup>11</sup> and othotoluidine<sup>1-12</sup> have been used as the reagents. These substances vary in their reactions with the pH of the urine,<sup>12</sup> ascorbic acid level,<sup>12-14</sup> presence of iodides and bromides,<sup>12</sup> yeast or pus cells.<sup>10</sup> Some investigators<sup>1-11-12</sup> have correlated the color reactions with quantitative erythrocyte counts of urinary sediments and claim that positive results occur only in the presence of a significant number of red blood cells. Others fail to find this correlation.<sup>15</sup> Endtz<sup>16</sup> has identified erythrocytes in the urinary sediment utilizing a benzidine stain. We found this technique to be slower acting and to give less intense color than the benzidine-nitroprusside method. The common practice of producing hemolysis of red blood cells by the addition of acetic acid to the sediment is unreliable, particularly in specimens containing preservatives.

To corroborate the varied appearance of red cells observed in submitted specimens, samples of normal blood were suspended in varying concentrations of sodium chloride with specific gravities of 1.004 to 1.040 with and without preservative tablets.\* Studies of the sediments from these solutions substantiate our opinion of a great variation in size and appearance of red blood cells in urine specimens of different specific gravities and salt concentrations. The sediments from suspensions containing the preservative tablet had a more constant form of erythrocyte than those without the preservative tablet. Although the tablet liberates formaldehyde in solution, no inhibiting reaction upon the stain was observed. The buffer and slight acidifying effect of the preservative tablet creates a condition favorable to staining.

Of particular interest was the finding that the granules of fine and coarse granular casts stain the same dark bluish-purple of red cells and red cell debris which was found in the sediment of red cell suspensions. This would tend to

\*Preservative tablet No. 4 Metropolitan Life Insurance Company specification contains: mg potassium acid phosphate, sodium benzoate, benzoic acid, Urotropin, sodium bicarbonate, mercuric oxide red.



indicate that the granules are particles of red blood cells which have been destroyed in the kidney and incorporated in the substance of the cast. This helps to explain the more serious implication of granular casts.

The efficacy of various preservative materials was evaluated by preparing suspensions of red cells in urine with preservative tablet, thymol, toluene, chloroform, boric acid, and formaldehyde. None of these materials inhibited the staining reaction, but only the formaldehyde and the preservative tablet satisfactorily preserved erythrocytes. From our suspension of erythrocytes in urine with one preservative tablet per ounce we obtained 100 per cent recovery of red blood cells even after seventy-two hours. This is greater than the 50 to 80 per cent recovery estimated by some investigators.<sup>1</sup> We have obtained identical counts per high power field and have had positive staining reactions on specimens that have stood at room temperature for as long as four to five months. There must be some breakdown of red cells in the kidney; however, as our findings on the nature of the granules of granular casts suggest.

*Normal Limits*—We were further interested in determining the number of red blood cells in the urinary sediment that may be considered within normal limits. Since the majority of urinalyses are on single voided specimens, we endeavored to correlate the results of quantitative studies with those that might be expected on sediments from random specimens. It would appear that a normal individual may excrete as many as 600,000 red blood cells per twelve-hour period.<sup>15, 1</sup> This number of erythrocytes suspended in 300 cc of urine, the average twelve-hour output of the quantitative studies, gave about 2 red blood cells per high power field by our standard technique. This technique consists of centrifugation of 15 cc of urine at 2,000 revolutions per minute for five minutes and examination of twenty high power fields of a drop of sediment placed beneath a  $\frac{7}{8}$  inch cover slip. Although the findings of single voided specimens may vary,<sup>18</sup> it would appear that the repeated presence of more than 2 red blood cells per high power field in the centrifuged sediment may indicate an increased loss of erythrocytes from one of the many sources of occult bleeding in the urinary tract. This estimate is supported by the laboratory findings in 3,000 consecutive single voided specimens from young men applying for employment. In this series 2,484 specimens were negative for erythrocytes. In sixty instances or only 2.0 per cent the specimens contained 2 to 3 red blood cells per high power field, and only twenty-three samples or 7 per cent contained 4 to 5 red blood cells per high power field. These values support our findings on the correlation of quantitative studies that the repeated excretion of more than 2 red blood cells per high power field may be significant.

The count per high power field obtained by adding the staining solutions to the sediment directly upon a slide is lower than that of an unstained sediment. To avoid dilution the reagents may be added to the sediment within the tip of the centrifuge tube, carefully mixed, recentrifuged, and the supernatant fluid decanted. The sediment thus prepared will have approximately the original dispersion of red blood cells. In performing quantitative counts the staining solution may be used in diluting to the desired volume in the Addis centrifuge tube.

## SUMMARY

A benzidine-nitroprusside stain for erythrocytes in the urinary sediment is described which we believe to be specific for red blood cells and fragments thereof. With this technique the red blood cells stain a deep blue purple and are easily distinguished from contaminants and other formed elements of urine.

The stain is based upon the pseudoperoxidase reaction of hemoglobin and benzidine. The nitroprusside may act as a catalyst.

The granules of fine and coarse granular casts take on the same bluish purple stain. This finding suggests fragments of red blood cells as the origin of the granules and helps explain the more serious implication of granular casts.

Erythrocytes may occur in the urinary sediment of normal individuals up to approximately 2 per high-power field. This level, determined by a conversion of the results of quantitative studies to the methods employed in analyzing single specimens, is supported by the results of our findings in 3,000 consecutive urinalyses in young men applying for employment. In this series, only 12 per cent of specimens contained more than 2 red blood cells per high power field.

The preservative tablet and formaldehyde are efficient agents for the preservation of erythrocytes. Thymol, toluene, chloroform, and boric acid are not satisfactory. None of these substances interfere with the stain for erythrocytes.

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## THE SPECTROPHOTOMETRIC DETERMINATION OF BLOOD pH

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PROBABLY the most frequently used methods for the determination of blood pH are those of Hastings and Sendroy,<sup>1</sup> Hawkins,<sup>2</sup> and Shock and Hastings.<sup>3</sup> In recent years the electrometric determination of pH has been brought to a high degree of accuracy under certain well controlled conditions especially by Nims.<sup>4</sup> However for purposes of clinical research the preceding visual colorimetric methods usually are considered adequate. There is one drawback which detracts from their usefulness—the observer must be experienced in the visual matching of colors. While this usually is not difficult with normal clear serum or plasma, slight amounts of hemolysis, lipemia or bilirubinemia often confuse the inexperienced observer. In addition there are certain individuals who have great difficulty in distinguishing the difference between density and hue of color.

It was with these difficulties in mind that an objective spectrophotometric method of determining blood pH was sought for. Evelyn has described such a method but there is no technical provision for comparison with other methods. Therefore it was decided to apply spectrophotometric measurements to the method of Hastings and Sendroy<sup>1</sup> in such a manner that comparison with the visual method could be made on the same sample.

The principle of this method depends on the fact that the color of the indicator, phenol red, is different in acid and alkaline solutions. At a given pH the relative amounts of these two colors determine the final color of the solution. These two colors absorb light of widely different wave lengths and can be measured independently in the same solution with a suitable spectrophotometer. Since the relative amounts of these two colors are dependent upon pH the measurement of these amounts constitutes an indirect measurement of pH.

*Reagents*—The reagents are those of Hastings and Sendroy. Detailed directions for their preparation are given elsewhere.\*

*Procedure*—Four milliliters of the adjusted dye saline mixture are placed in a colorimeter tube and covered with a small amount of paraffin oil. Two tenths milliliter of serum or 0.4 ml. of blood are then added and the mixture is stirred gently with a glass rod. In the case of blood the tube is centrifuged to throw down the cells. The blank is prepared in a similar fashion substituting saline for dye saline. The optical density of the sample is determined at wave lengths 565 and 420 m $\mu$  after which the temperature of the solution is recorded.

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Letters and Van Slyke: Quantitative Clinical Chemistry, vol. II, p. 736, 1932.

*Calculation*—The ratio of optical density at 565  $m\mu$  to optical density at 420  $m\mu$  has been determined for a number of solutions of known pH (Fig 2) For easy reference it is convenient to prepare such a graph from the data supplied by Evelyn<sup>5</sup> After having calculated the ratio, read the pH from the graph This value is the uncorrected pH The true pH is obtained by applying the temperature correction<sup>6</sup>

$$\text{True pH} = \text{Uncorrected pH} - (0.42 - 0.1 T^{\circ} \text{C})$$

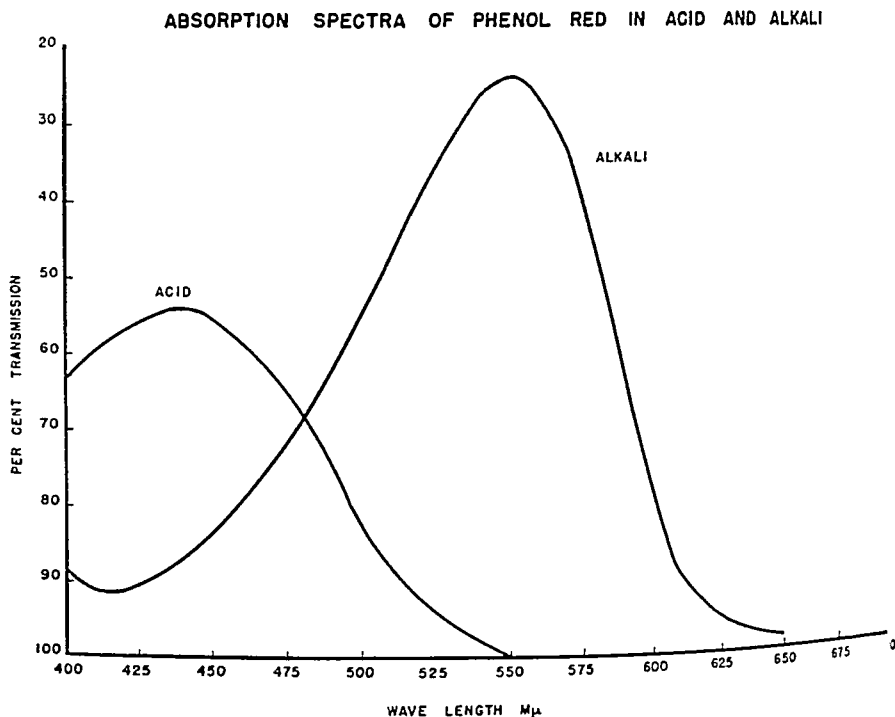


Fig 1

*Experimental*—The absorption spectrum of phenol red in both acid and alkaline solution was determined with the Beckman spectrophotometer To a four milliliter portion of dye-saline solution were added 0.2 ml portions of strong acid or alkali The transmission of the resulting solutions was determined at various wave lengths (Fig 1) It is seen that measurements of the two colors are best taken at wave lengths 565 and 420  $m\mu$  to avoid undue interference

In order to check the data supplied by Evelyn for the relation of pH to the ratio of optical densities in dilute solutions of phenol red, a series of Sorensen's M/15 phosphate buffers was prepared,\* the pH being accurately adjusted electrometrically To four milliliter portions of the adjusted dye-saline solution were added 0.2 ml portions of the buffer The optical densities of the resulting solutions were determined at wave lengths 565 and 420  $m\mu$  at 35°C

\*By Mr J Earle Adler

The ratio of the densities was plotted against pH resulting in the curve shown in Fig. 2. As was expected when  $\log$  ratio was plotted against pH a straight line resulted. These data are in good agreement with the data of Evelyn.

To compare the visual with the spectrophotometric method the pH standards described by Hastings and Sendroy were prepared. To insure stability these standards were prepared in sodium borate and boric acid instead of hydrochloric acid and sodium hydroxide. Test tubes of the same size were selected for use in the Coleman Junior spectrophotometer. The comparison was made as follows. To four milliliters of adjusted dye saline solution in a colorimeter tube were added 0.2 ml. of serum. The usual blank was prepared and the tubes were warmed to 39° C., after which comparison was made with the visual standards.

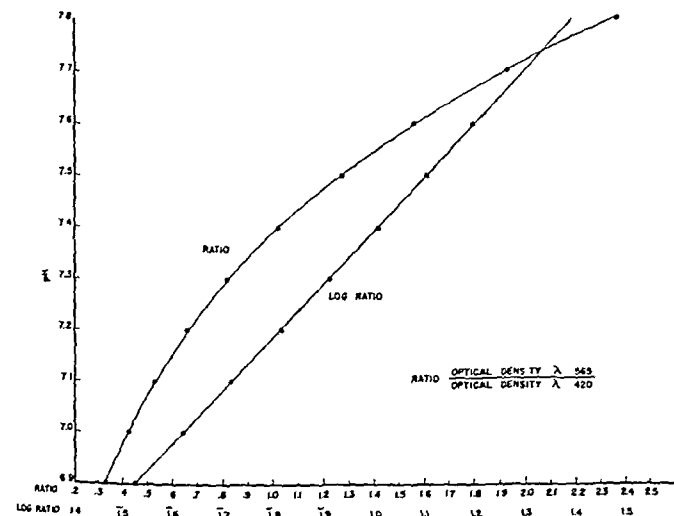


Fig. —The relation between pH and the ratio of the optical densities at wave lengths 65 and 420 millimicrons

The tubes were then allowed to cool to room temperature and were placed in the spectrophotometer where the optical densities at 565 and 420  $m\mu$  were determined. The ratio of optical densities was calculated the corresponding pH read off the graph, and the temperature correction applied. The sera used were taken from both normal and pathologic subjects. The comparison of the two methods is seen in Table I along with other pertinent data. In some cases two or more observers participated in the visual comparison. These data indicate that the spectrophotometric method is at least as acceptable as the visual. In addition there is the added advantage that the personal error inherent in the

TABLE I COMPARISON OF VISUAL AND SPECTROPHOTOMETRIC DETERMINATION OF pH

SUBJECT	OPTICAL DENSITY $\lambda = 565 \text{ m}\mu$	OPTICAL DENSITY $\lambda = 420 \text{ m}\mu$	RATIO	UNCORRECTED pH	T° C	CORRECTED pH	VISUAL pH			DIFFERENCE	SERUM CO CONTENT (VOL %)
							OBSERVER				
							1	2	3		
1 (Schizophrania)	377	2291	1.65	7.63	32.0	7.53	7.50	7.50		03	63
2 (Betahydroxybutyric acid tolerance test)	374	2200	1.70	7.64	29.5	7.53	7.50			03	58
3 (Betahydroxybutyric acid tolerance test)	369	2200	1.66	7.63	33.0	7.54	7.53			01	57
4 (Normal)	392	2384	1.64	7.62	25.5	7.46	7.45	7.54	7.54	01*	51
5 (Diabetic acidosis)	310	2840	0.91	7.30	25.0	7.13	7.15	7.15	7.15	02	25
6 (Pancreatic fibrosis)	352	2007	1.57	7.65	33.0	7.57	7.59			02	53
7 (Pancreatic fibrosis)	342	2384	1.43	7.56	33.0	7.47	7.45			02	20
8 (Diabetic acidosis)	2403	2798	0.86	7.33	28.0	7.19	7.20			01	18
9 (Diabetic acidosis)	1956	2915	0.67	7.21	30.0	7.09	7.10	7.10		01	113
10 (Pancreatic fibrosis)	323	1973	1.64	7.62	34.0	7.54	7.55			01	63
11 (Pyloric stenosis)	406	1756	2.31	7.79	33.5	7.71	7.73	7.71	7.71	00	27
12 (Pancreatic fibrosis)	347	1922	1.80	7.67	32.0	7.57	7.53	7.53		04	
13 (Diabetic acidosis)	297	2557	1.16	7.46	30.0	7.34	7.35			01	
14 (Normal)	403	2997	1.61	7.60	27.0	7.45	7.45			00	
15 (Normal)	382	2291	1.66	7.63	27.0	7.48	7.50			02	
16 (Normal)	301	2596	1.16	7.46	32.0	7.46	7.45			00	
17 (Normal)	344	2676	1.28	7.51	32.0	7.41	7.40			00	
18 (Normal)	374	2882	1.29	7.51	30.0	7.39	7.40			01	
Average difference										01.4	

\*Because of the normal CO content it would seem that the value 7.15 is the more nearly correct one

visual method is eliminated and this at the slight expense of the preparation of a graph and the selection of several colorimeter tubes. However, the errors of the colorimetric method *per se* are retained.

#### SUMMARY

An objective spectrophotometric method for the determination of blood pH is described. A comparison of the visual and spectrophotometric methods is presented showing an average difference of between .01 and .02 pH units.

Critical acknowledgement is made to Dr. Alexis F. Hartmann for advice and criticism during the course of this work and in the preparation of this paper.

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# A MICROMETHOD FOR THE DETERMINATION OF SERUM STREPTOMYCIN LEVELS\*

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A MICROMETHOD for the assay of streptomycin in whole blood has been described by Foigacs and Kuceia<sup>1</sup> using agar plates seeded with a variant strain of *Bacillus subtilis*, Cohn *emend* Piasmowski, blood levels are estimated from the zones of inhibition produced by 0.1 ml aliquots of oxalated blood. Although it is a microtechnique for each determination, the construction of standard curves requires larger amounts of blood. Individual blood idiosyncrasies require that a separate standard curve be constructed for each patient. Furthermore, accuracy in the method requires maintenance of a uniform distribution of cells during the entire procedure, which is very difficult in bloods with low hematocrit values and elevated sedimentation rates. Since streptomycin does not enter the cells<sup>2</sup> and since the just mentioned difficulties seemed to stem from the presence of cells, it seemed desirable to modify the method for use with serum alone. Because of the decreased variables it was thought that one master standard curve might be generally applicable. The following technique was therefore developed.

*Preparation of the Standard Curve*—The serum from 12 ml of blood was required to determine the thirteen points on the curve. Final concentrations of 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, 25, and 35  $\mu\text{g}$  of streptomycin were prepared as described by Foigacs and Kuceia,<sup>1</sup> with the exception that serum was substituted for oxalated blood. Agar plates were seeded with *B. subtilis* as in the original method.<sup>1</sup> The serum-streptomycin mixture was drawn up into a 0.1 ml serologic pipette with the aid of a Guthrie pipette controller.† The meniscus was lowered to one of the graduations, and the tip was wiped dry. By careful turning of the pipette controller screw, 0.1 ml was forced onto the pipette tip and touched to the agar plate which was resting on a level surface. This was repeated on each of five plates. There is space on each plate for four zones, so that each set of five plates was used for four different concentrations. The plates were incubated overnight at 30° C. The diameters of the zones of inhibition were then read with a millimeter rule. The mean of the five readings for each concentration was recorded. This procedure was carried out on thirty individual sera. The mean value for each point was obtained and plotted against micrograms of streptomycin, as shown in Fig. 1.

The statistical data are listed in Table I. At only two points, namely 3 and 15  $\mu\text{g}$  per milliliter, did the number of observations falling outside the

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†Obtained from Eimer and Amend New York N Y.



range of mean  $\pm 2\sigma$  exceed 5 per cent. Sera from all age groups (newborn to adult) were used, and no significant difference between sera was found. Thus a master standard curve such as this can be used for all determinations.

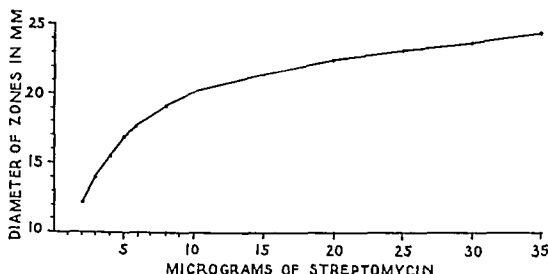


Fig. 1.—Standard curve computed from thirty sera.

TABLE I. STATISTICAL DATA—SERUM STANDARD CURVE

STREPTOMYCIN (MG PER ML.)	NUMBER OF OBSERVATIONS	RANGE (DIAMETER OF ZONE IN MM.)	MEAN (DIAMETER OF ZONE IN MM.)	STANDARD DEVIATION	NUMBER OF OBSERVATIONS OUTSIDE RANGE OF MEAN $\pm$ STANDARD DEVIATIONS
2	27	11.4—13.2	12.1	39	1
3	28	13.2—14.8	14.0	44	0
4	30	14.4—16.6	15.6	52	1
5	30	15.8—17.4	16.8	38	3
6	30	17.0—18.6	17.7	46	0
7	30	17.8—19.0	18.4	36	0
8	28	18.6—19.8	19.1	40	0
10	29	19.4—20.8	20.0	39	1
15	30	20.2—22.4	21.3	50	2
20	20	21.4—23.0	22.3	47	0
25	30	22.2—23.8	23.0	43	0
30	27	23.0—24.4	23.7	42	0
35	21	23.8—25.2	24.5	38	0

*Determination of Serum Streptomycin Level*—Capillary blood is collected. The collecting tube is made either from a three inch length of glass tubing with one end sealed, or from a 7.5 by 95 mm shell vial. The open end is drawn out to a capillary tip and sealed. The wide portion of the tube is held over the flame and an air vent is quickly produced in the softened glass by the expanding air within the sealed tube. For the collection of blood the capillary tip is broken off near its base. The finger or heel is punctured so that a free flow of blood is obtained. The tube is held in as nearly vertical a position as possible and the blood readily flows into the tube and down to the base. For each determination 0.3 ml. or a 1.5 cm. column of blood is adequate. The tubes are stood upright until the blood is clotted, then the tube is filed off just above the surface of the blood. The clot is broken up with an applicator stick and the tube is centrifuged at 1500 to 2000 revolutions per minute for fifteen minutes. As

described, 0.5 ml serum is then drawn up into a 0.1 ml pipette and a drop of 0.1 ml is applied to each of five prepared plates. The plates are incubated overnight at 30° C. The mean of the five zones is converted to micrograms of streptomycin by means of the standard curve.

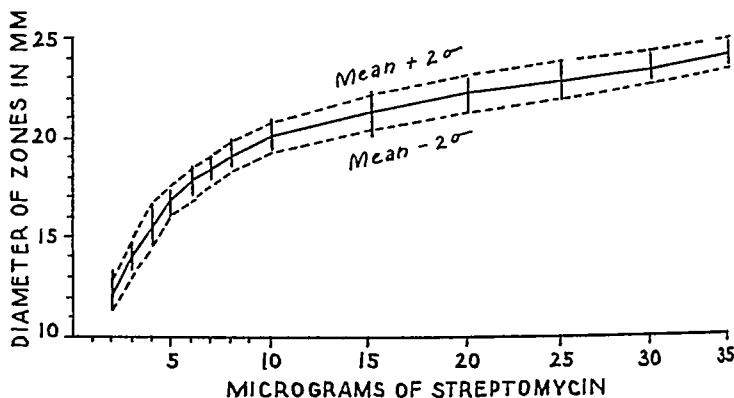


Fig 2—Chart showing expected variations in streptomycin values determined from given zone diameters. Solid curve represents the standard curve as in Fig 1. Dotted curves represent value of the mean +2 standard deviations and the mean -2 standard deviations. Vertical lines represent the range of zone diameter means obtained at each serum concentration of streptomycin. There are very few determinations falling outside the range of  $\pm 2$  standard deviations.

Fig 2 is a graphic representation of the range of error to be anticipated in the method. Rarely does the spread of observations exceed the range of 2 standard deviations. It can be seen that the least variation in estimated streptomycin level for a given zone diameter is to be found in that portion of the curve from 2 to 6  $\mu$ g of streptomycin, where for clinical purposes the least deviation is desirable.

For good results, the following precautions should be observed:

(1) Media must be prepared with meticulous constancy, for slight variations in ingredients may markedly affect the activity of streptomycin.

(2) The pH of the medium must be adjusted to 7.2 shortly before the plates are poured.

(3) Results are best when flat-bottomed Petri dishes are used\*. It is important that the 10 ml of medium be measured carefully when plates are poured.

(4) Since dust particles distort the drops of serum, the room should be dust-free during the entire procedure.

(5) The spore suspension is remarkably stable and is usually reliable for several months. However, it may become contaminated, with a resulting change in sensitivity. If this method is being used routinely over a long period of time, it is well to run a weekly or fortnightly standard curve to insure reproducibility.

Even with the increased variation at higher levels, the method is sufficiently exact for clinical purposes. It must be pointed out, however, that without modifications the method is not reliable for use in a patient receiving both penicillin and streptomycin, since the strain of *B. subtilis* used is moderately sensitive to penicillin.

\*Pressed glass Petri dishes were obtained from Corning Glass Company, Corning, N. Y.

## SUMMARY

A method is described for estimating streptomycin in serum. It uses only 0.3 ml. of blood, and therefore is particularly applicable for use among infants and children. It requires no unusual or expensive equipment and the laboratory techniques are easily acquired and carried out.

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## A METHOD FOR THE QUANTITATIVE DETERMINATION OF BILIRUBIN IN URINE

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SEVERAL procedures for the quantitative determination of bilirubin in urine have been described. Most of these have utilized the principle of adsorption of bilirubin from urine on insoluble barium or calcium salts. Bilirubin is eluted from the adsorbate and some characteristic color reaction is used to determine the concentration. Methods employing these principles have been described by Hunter,<sup>1</sup> Godfried,<sup>2</sup> and With<sup>3</sup> among others.

Our efforts to use such techniques for the quantitative determination of bilirubin proved disappointing for several reasons.

First, we were unable to effect complete removal of bilirubin from icteric urine. Pure bilirubin added to normal urine could be removed completely from solution by addition of barium chloride and centrifugation of the adsorbate, but quantitative removal of naturally occurring bilirubin from icteric urine could not be achieved by the same technique.

Second, elution of bilirubin from the barium sulfate bilirubin complex was incomplete after centrifugation.

Finally, the most disturbing problem arose in connection with the color development of the eluted bilirubin. The previously mentioned authors employed some modification of the Ehrlich diazo reaction. This reaction gives a characteristic red color with bilirubin. On diazotization, many urines develop a reddish brown color which masks the color of diazotized bilirubin. With<sup>3</sup> tried to overcome this difficulty by extraction of the diazotized bilirubin with chloroform, but he reported that he was unsuccessful in obtaining quantitative extraction.

Some investigators have tried to estimate bilirubin in urine by direct diazotization without preliminary adsorption. Goodson and Sheard<sup>4</sup> have published such a procedure. In their procedure no means of obviating the interference due to the diazotizable nonbilirubin chromogenic material in urine is offered.

Our attempts to determine bilirubin in urine by direct diazotization in simple alcoholic solution gave promising results when highly icteric urine was tested. However, normal dark urines free from bilirubin developed a red brown color upon diazotization. This red-brown color absorbed a significant amount of light when read in a photometer at a wave length suitable for diazotized bilirubin. A direct diazotization method, therefore, seemed to require some means of avoiding the interference of this diazotizable nonbilirubin chromogenic material.

It seemed feasible to avoid this interference by application of the Vierordt principle<sup>5</sup> for determining individual concentrations of pigments in a mixture. This requires that the mixture be read at two wave lengths that the ratios of the optical densities at both wave lengths for each pigment in pure solution be known and that these ratios be constant over the range of concentrations to be tested. Gibson and Evelyn<sup>6</sup> have applied this principle to the photometric determination of Evans blue in serum in the presence of hemoglobin. Engstrom and Mason and others have employed it for the photometric determination of 17 ketosteroids in urine.

Employing this principle we have developed a procedure for the determination of bilirubin in urine which overcomes interference from nonbilirubin material.

#### Reagents —

#### METHOD

(1) Alcohol 95 per cent ethanol U.S.P.

(2) Diazo reagent A. Dissolve 1 Gm sulfanilic acid in 10 ml concentrated hydrochloric acid in a liter volumetric flask and dilute to mark with water (keeps indefinitely.)

Diazo reagent B. Dissolve 0.5 Gm sodium nitrite chemically pure in water and make to 100 milliliters. Store solution in refrigerator and discard when it develops discernible color.

Diazo reagent for use. Mix fresh prior to determination 10 ml diazo reagent A and 0.3 ml diazo reagent B.

*Procedure* — One milliliter of urine (as voided or diluted\*) is measured into a colorimeter tube (19 by 150 mm). Eight milliliters of alcohol and 1 ml of freshly prepared diazo reagent are successively added. Mix. Allow thirty minutes for color development then add 0.25 ml concentrated hydrochloric acid and mix. Read per cent transmission in photometer† at wave lengths of 575 and 450  $m\mu$  with photometer adjusted to read 100 per cent transmission with alcohol blank at each respective wave length.

Convert the transmission readings at each wave length to corresponding optical densities.

Optical density equals  $2 \text{ minus } \log T$  where  $T$  equals per cent transmission. The optical density of bilirubin is then calculated from the equation  $Y_7 = 1.05M_7 - 0.202M_4$ , where  $Y_7$  equals optical density of bilirubin at 575  $m\mu$ ,  $M_7$  equals observed optical density of diazotized urine at 575  $m\mu$ ,  $M_4$  equals observed optical density of diazotized urine at 450  $m\mu$  and where the numerical constants have been derived experimentally as given elsewhere in the text.

Given  $Y_{75}$  the concentration of bilirubin equals  $K$  times  $Y_7$  where  $K$  is the calibration factor obtained as described below.

#### Standardization With Pure Bilirubin —

Stock standard (10 mg per cent). Dissolve 10 mg of pure bilirubin‡ in chloroform and make to a volume of 100 milliliters.

\*In clinical testing the urine is diluted to 100 ml per hour if the urine flow is not already greater than that.

†Photometer readings are made on the Coleman Jr. Clinical Spectrophotometer model 6A.

‡Eastman Kodak Bilirubin No. 101.

Dilute standard (1 mg per cent) Dilute 10 ml of the stock standard to 100 ml with alcohol Measure 0.5, 1, 2, 3, 4, and 5 ml of the 1 mg per cent standard into colorimeter tubes These tubes correspond respectively to 0.5, 1, 2, 3, 4, and 5 mg per cent Make each to a volume of 8 ml with alcohol Add 1 ml of water to each and mix To each add 1 ml diazo reagent, mix, allow color to develop thirty minutes, then add 0.25 ml concentrated hydrochloric acid Mix and read at 575 m $\mu$  against alcohol blank set at 100 per cent transmission A transmission-concentration curve may be plotted on semilog paper or a conversion factor may be calculated from the corresponding optical densities according to the formula  $K = \frac{C}{D}$  (where K is the conversion factor C is concentration in milligrams per cent and D is optical density) for that range of concentration where K is constant

Standardization performed in the preceding manner has yielded a K value of 6.2 for concentrations up to 5 mg per cent of bilirubin Thus,  $Y_s$ , times 6.2 equals concentration of bilirubin in milligrams per cent

#### SPECTROPHOTOMETRIC STUDIES

In the development of the method, certain spectrophotometric studies were carried out Bilirubin solutions, icteric urine, and normal urine were treated according to the described method with certain variations which will be noted The spectral transmittance curves were then determined \* They are reproduced in Figs 1 to 3

The preparation of the material for the study was made as follows

Preparation 1 Bilirubin solution (equivalent to 2 mg per cent in urine) treated with diazo reagent and not subsequently acidified (*Curve 1*, Fig 1)

Preparation 2 Bilirubin solution (same as in Preparation 1) treated with diazo reagent and then acidified (*Curve 2*, Fig 1)

Preparation 3 Normal urine treated with diazo blank† and not subsequently acidified (*Curve 3*, Fig 2)

Preparation 4 Normal urine treated with diazo reagent and not subsequently acidified (*Curve 4*, Fig 2)

Preparation 5 Normal urine treated with diazo blank† and then acidified (*Curve 5*, Fig 2)

Preparation 6 Normal urine treated with diazo reagent and then acidified (*Curve 6*, Fig 2)

Preparation 7 Icteric urine treated with diazo blank† and not subsequently acidified (*Curve 7*, Fig 3)

Preparation 8 Icteric urine treated with diazo reagent and not subsequently acidified (*Curve 8*, Fig 3)

Preparation 9 Icteric urine treated with diazo blank† and then acidified (*Curve 9*, Fig 3)

\*On a General Electric recording spectrophotometer at the Stamford Research Laboratories of the American Cyanamid Co

†Hydrochloric acid 15 per cent (15 ml concentrated hydrochloric acid in 100 ml solution)

Preparation 9 Icteric urine treated with diazo blank† and then acidified (*Curve 10*, Fig 3)

*Curve 1* represents the spectral transmittance of the red (nonacidified) azobilrubin, the derivative commonly employed for quantitative bilirubin determinations in serum. This red pigment has its absorption maximum at 530 to 540 millimicrons. If the red azobilrubin is strongly acidified with hydrochloric acid, it is at once converted to a purple pigment\*. *Curve 2* shows the spectral transmittance of this pigment. The purple (acidified) azobilrubin has

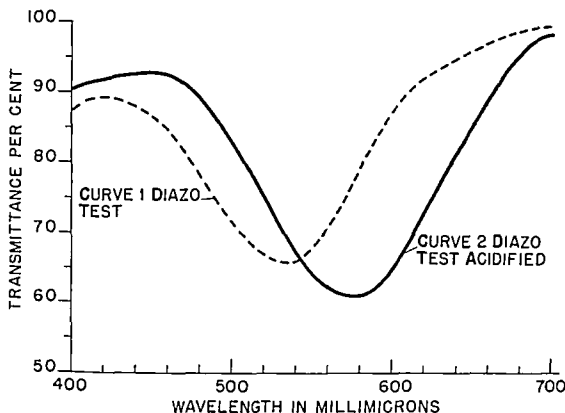


Fig 1

its absorption maximum at 570 to 580 millimicrons. Since *Curves 1* and *2* represent equal amounts of bilirubin it is seen that the purple pigment absorbs more light at 575  $m\mu$  than the red pigment at 530 millimicrons. This alone suggests that the purple (acidified) azobilrubin is the more sensitive derivative for photometric analysis.

Fig 2 presents the curves obtained using normal urine presumably bilirubin free. *Curve 4* shows the transmittance of a pigment developed by diazotization and *Curve 6* shows the change in transmittance produced upon acidification. *Curve 3* is the blank for *Curve 4* and *Curve 5* is the blank for *Curve 6*.

*Curve 4* has no maximum absorption band in the visible spectrum while *Curve 6* has a broad maximum absorption band from 440 to 480 millimicrons. Both preparations show significant absorption at the wave lengths at which azobilrubin may be read that is 530  $m\mu$  for the red or 575  $m\mu$  for the purple pigment. The presence of the interfering substances in normal urine clearly indicates the need for a means of correction in methods employing direct diazotization. For convenience we designate this interfering material as a chromogen.

† The conversion of red azobilrubin to a purple pigment by acidification with hydrochloric acid has been known for some time and in fact, I attributed to both Ehrlich and Prosser (original reference not available) as cited by Müller.

Fig 3 presents the transmittance curves of icteric urine treated similarly to the normal urines of Fig 2. *Curve 8* represents the transmittance characteristics of red azobilinubin modified by the presence of the unacidified diazo-

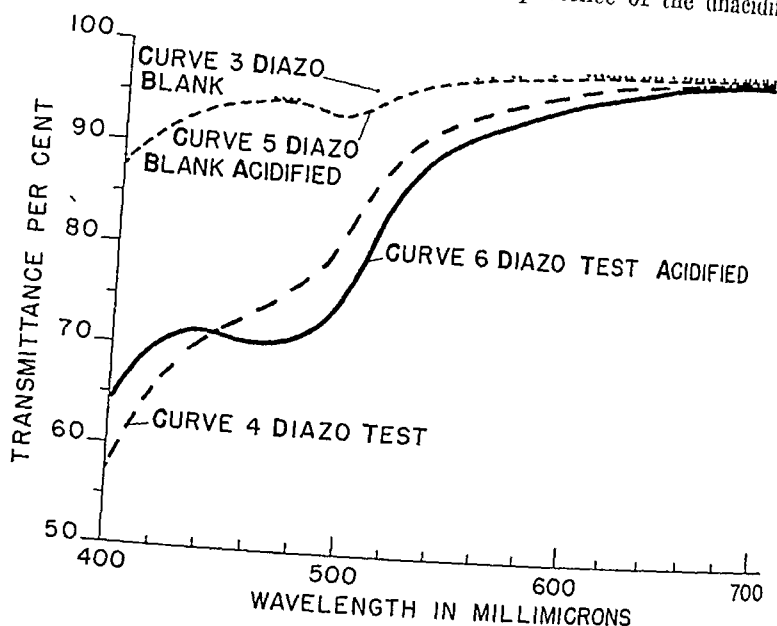


Fig 2

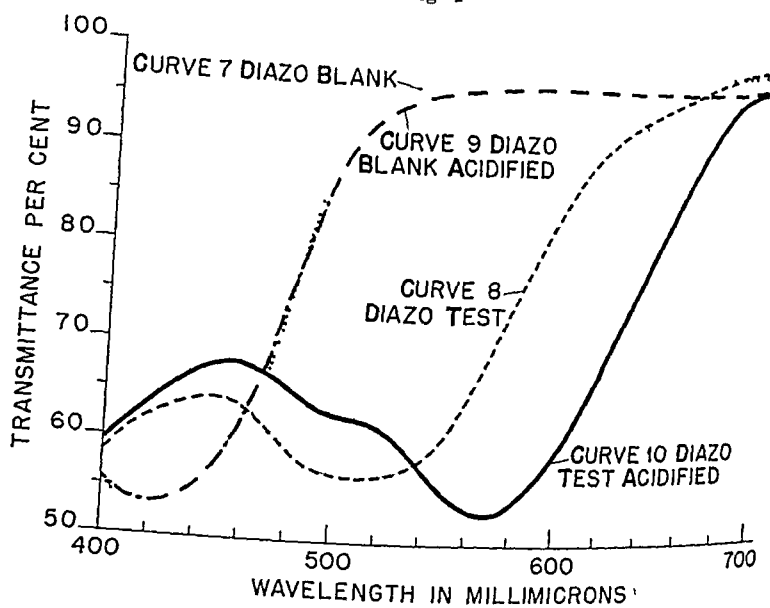


Fig 3

tized  $\lambda$ -chromogen. This shows a shift of the absorption maximum from 530 to 540  $m\mu$  for pure bilirubin (*Curve 1*, Fig 1) to 490 to 530 millimicrons. This curve is smooth since  $\lambda$ -chromogen has no absorption maximum in the visible



spectrum *Curve 7* is the blank for *Curve 8*. *Curve 10* presents the transmittance characteristics of purple (acidified) azobilirubin (*Curve 2* Fig. 1) modified by the presence of acidified diazotized x chromogen. *Curve 10* shows that the absorption maximum is neither shifted nor significantly broadened but there is a distinct depression in the otherwise smooth curve at 490 to 510  $m\mu$  apparently the effect of the absorption due to acidified diazotized x chromogen. These properties emphasize the superiority of the purple (acidified) azobilirubin for photometric analysis. *Curve 9* is the blank for *Curve 10*.

#### DERIVATION AND APPLICATION OF CORRECTION EQUATION

The general form of the equation which applies for the photometric determination of the individual pigments of a two component mixture is derived as follows

- Let  $\lambda$  = optical density of  $\lambda$  substance at wave length  $a$   
 $\lambda_b$  = optical density of  $\lambda$  substance at wave length  $b$   
 $Y$  = optical density of  $Y$  substance at wave length  $a$   
 $Y_b$  = optical density of  $Y$  substance at wave length  $b$   
 $M$  = optical density of a mixture of  $\lambda$  and  $Y$  at wave length  $a$   
 $M_b$  = optical density of a mixture of  $\lambda$  and  $Y$  at wave length  $b$

$$(1) \quad R = \frac{Y}{\lambda_b} \qquad (2) \quad R_y = \frac{Y}{\lambda_b}$$

If substances  $\lambda$  and  $Y$  do not react chemically with one another the following holds

$$(3) \quad M = \lambda + Y \qquad (4) \quad M_b = \lambda_b + Y_b$$

From (2) and (3)

$$(5) \quad M = \lambda + R_y Y$$

And from (1) and (4)

$$(6) \quad M_b = \frac{\lambda}{R} + Y$$

Solving equations (5) and (6) for  $Y_b$  we obtain

$$(7) \quad Y_b = \frac{R M_b - M}{R - R_y}$$

An inspection of the spectral transmittance curves in Figs. 1 to 3 indicates two suitable wave lengths are 575 and 450 millimicrons. If we let  $Y$  represent bilirubin,  $X$ , the interfering chromogen at 450  $m\mu$ , and  $b$  575  $m\mu$ , then equation (7) can be rewritten

$$(8) \quad Y_b = \frac{R M_b - M_x}{R - R_x}$$

To apply the formula for use the numerical values of  $R_x$  and  $R_y$  at 450 and 575  $m\mu$  must be derived. Values for  $R_x$  were obtained by diazotizing normal bilirubin free urines by the method given previously and finding the optical densities at 450 and 575 millimicrons

$$R = \frac{\lambda_a}{\lambda_b} = \frac{\text{optical density of x chromogen at 450 } m\mu}{\text{optical density of x chromogen at 575 } m\mu}$$

TABLE I DETERMINATION OF EQUATION CONSTANTS

	NUMBER OF DETERMINATIONS	MEAN VALUE	STANDARD DEVIATION OF MEAN
$R_x = \frac{X_{1.0}}{X_{5.5}}$	49	5.19	$\pm .031$
$R_y = \frac{Y_{1.0}}{Y_5}$	61	0.25	$\pm .006$

TABLE II ADDITION OF BILIRUBIN TO URINE

CASE	$M_{5.5}$ OBSERVED	$M_{1.0}$ OBSERVED	$Y_5$ COMPUTED	$Y_{5.5}$ ADDED	$\Delta Y_5$ COLUMN 4 MINUS COLUMN 5
1	125	377	055	043	+ 012
2	194	432	117	102	+ 015
3	324	475	234	222	+ 012
4	083	187	049	051	- 002
5	131	426	051	051	0
6	111	328	051	051	0
7	161	420	084	084	0
8	155	367	089	084	+ 005
9	212	758	070	084	- 014
10	215	620	101	110	- 009
11	177	420	101	110	- 009
12	229	783	082	110	- 028
13	149	177	120	116	+ 004
14	146	177	117	116	+ 001
15	155	187	125	116	+ 009
16	155	229	117	116	+ 001
17	161	229	123	116	+ 007
18	149	116	133	116	+ 017
19	155	215	120	116	+ 004
20	158	237	118	116	+ 002
21	071	177	039	037	+ 002
22	105	347	040	037	+ 003
23	066	155	038	037	+ 001
24	056	116	036	037	- 001
25	076	248	030	037	- 007
26	071	208	033	037	- 004
27	076	237	032	037	- 005
28	111	181	080	070	+ 010
29	137	337	076	070	+ 006
30	111	168	083	070	+ 013
31	108	131	087	070	+ 017
32	111	244	068	070	- 002
33	111	222	072	070	+ 002
34	119	244	076	070	+ 006
35	097	184	065	077	- 012
36	111	229	071	077	- 006
37	128	347	064	077	- 013
38	197	276	151	158	- 007
39	190	240	151	158	- 008
40	197	284	150	158	- 008
41	194	276	148	158	- 010
42	208	282	141	158	- 017
43	347	284	307	323	- 016
44	342	218	316	323	- 007
45	357	342	306	323	- 017
46	347	201	323	323	0
47	347	256	311	323	- 012

\* $\Delta Y_5$ : The computed minus the expected optical density of the added amount of bilirubin that is column four minus column five.

Summary of data in Table II. Mean deviation ( $\Delta Y_5$ ) regardless of sign 00.0 mg per cent. Average per cent recovery of added bilirubin 99.7 per cent.

Values for  $R_x$  were similarly determined with pure bilirubin solutions. Table I shows the summarized data for the determinations of  $R_x$  and  $R_y$ .

Since  $R_x = 5.19$  and  $R_y = 0.25$  substitution of these values in equation (8) gives the final equation

$$(9) \quad Y_{75} = \frac{5.19 Y_7 - M}{5.19 - .25} = 1.05M_{75} - .202M_{450}$$

where  $Y_7$  equals the optical density of the bilirubin component of the mixture at 575  $m\mu$ ,  $M$  equals the optical density of the mixture of bilirubin and urobilinogen at 575  $m\mu$  and  $M_{450}$  equals the optical density of the same mixture at 450 millimicrons.

To determine the reliability of the equation and the method recoveries of bilirubin added to normal (bilirubin free) urine were carried out. The computed optical densities in these mixtures ( $Y_{75}$ ) were compared with the optical densities of the same amounts of pure bilirubin determined simultaneously. Since precise amounts of bilirubin cannot be added conveniently to urine directly, the additions were accomplished by dissolving the desired amount of bilirubin in the alcohol used as the diluent in the method.

Table II presents a summary of these data.

The differences between the calculated and expected optical densities ( $Y_{75}$  of Table II) were small and were random with respect to sign. The average recovery was 99.7 per cent. We conclude that equation (9) introduces no significant theoretical error and is therefore valid and applicable for the calculation of the optical density of bilirubin mixed with urobilinogen according to our method.

The arithmetic mean of the optical density differences (column six Table II) was 0.0075 regardless of sign and this optical density is equivalent to approximately 0.04 mg per cent of bilirubin. An error of this order of magnitude is of little significance.

In order to show the order of magnitude of urobilinogen we have presented in Table III data obtained from a representative group of icteric urines. These results show that urobilinogen was encountered in all of

TABLE III RELATIVE PROPORTIONS OF BILIRUBIN AND UROBILINOGEN IN ICTERIC URINE

CASE	DILUTION FACTOR	OBSERVED		COMPUTED		RELATIVE PROPORTION OF X AND Y (%)		EXPRESSED AS MG % BILIRUBIN	
		$M_{450}$	M	$X$	$Y \dagger$	$X$	$Y$	$X$ CHROMO GEN X	TRUE BILI RUBIN Y
1	5	40	562	0.30	332	8	92	93	10.30
	2	523	721	0.72	649	10	90	90	8.04
3	1	307	377	0.3	324	14	86	33	2.01
4	5	248	205	0.40	165	20	80	1.25	5.10
5	1	796	577	132	445	23	77	82	2.76
6	1	678	367	110	207	30	70	68	1.59
7	1	409	174	0.74	100	43	57	46	.62
8	1	319	119	0.38	061	49	51	36	.38
9	1	367	119	0.68	051	57	43	42	.32
10	1	384	111	0.72	039	65	35	45	.24

$Y_{75} = M_5 - Y_{15}$

$\dagger Y_{15}$  computed from equation (9)

these specimens and in amounts accounting for from 8 to 65 per cent of the observed optical density at 575 millimicrons. It is evident from the data that the correction for  $\lambda$ -chromogen is essential for the true bilirubin values.

#### MISCELLANEOUS DETAILS OF TECHNIQUE

Certain points of technique were investigated to determine conditions for optimum color development and reproducibility. The choice of alcohol as a diluent was based on the fact that the substitution of water for 95 per cent ethanol in the described method results in a diminution of optical density of diazotized icteric urine at both 450 and 575 millimicrons. An example is given in Table IV.

TABLE IV WATER VS 95 PER CENT ETHANOL AS THE DILUENT

DILUENT	OPTICAL DENSITY	
	575 m $\mu$	450 m $\mu$
Distilled water	268	168
95% Ethanol	337	252

The prescribed order of addition of reagents was found superior to other orders tried. Data in Table V show that each modification resulted in reduction of optical density at 575 millimicrons.

In the described method, thirty minutes are allowed for color development before the addition of acid because the color reaction changes little after

TABLE V ORDER OF ADDITION OF REAGENTS

ORDER OF ADDITION	OPTICAL DENSITY (575 m $\mu$ )		
	SOLUTION 1	SOLUTION 2	SOLUTION 3
1, 2, 3, 4, 5	350	--	--
1, 3, 4, 2, 5	310	--	--
1, 2, 3, 4, 5	--	314	633
1, 2, 3, 5, 4	--	233	475

- 1 Urine (or in case of solutions 2 and 3 bilirubin in 95 per cent ethanol)
- 2 95 per cent ethanol
- 3 Diazo reagent.
- 4 Color development period (thirty minutes)
- 5 Concentrated hydrochloric acid 0.25 milliliter

twenty-five minutes and is maximal at thirty minutes. Although the purple (acidified) azobilirubin is quite stable, there is no advantage in delaying the reading. The data concerning the rate of color development and the stability of the color are shown in Table VI. Pure bilirubin was used for these determinations.

TABLE VI RATE OF COLOR DEVELOPMENT

Minutes after addition of diazo reagent	5	10	15	20	25	30	35	45	75
Minutes after addition of acid*							5	15	45
Optical Density									
Solution 1	187	211	229	240	250	256	315	315	319
Solution 2	337	417	450	473	493	500	613	623	655

\*Acid added thirty minutes after diazo reagent.

In order to avoid variations due to rate of urine flow and to obviate turbidities in concentrated specimens it was found desirable to collect the urine over a timed period. The urine is then diluted to a total volume equal to 100 ml per hour. After mixing 1 ml of this diluted specimen is used for analysis. This dilution obviates turbidities and makes milligrams per 100 ml equivalent to milligrams per hour. For urine flow above 100 ml per hour appropriate correction must be made if the results are to be expressed in units of time. If the urine is turbid even after this dilution a portion should be centrifuged before taking the 1 ml for analysis. Highly icteric urines will require even greater dilutions than 100 ml per hour. Correction must be made for the further dilution in calculating the concentration of bilirubin or for expressing the amount of bilirubin per unit time.

Collection of specimens in stoppered brown bottles and prompt analysis are recommended to avoid loss of bilirubin as reported by With<sup>3</sup>. We have found losses of 14, 15, and 50 per cent of bilirubin from three icteric urine specimens held twenty-four hours in the refrigerator in stoppered brown bottles.

#### INTERFERING MATERIALS

We have investigated the possible interference with the procedure by certain common medications. Urine specimens were collected from patients receiving Solu B\* and penicillin. Neither of these substances was found to have interfering properties as shown by the  $\chi^2$  values presented in Table VII Groups I and II.

Sulfathiazole and sulfadiazine in amounts of 25 mg per cent do not interfere with the test as shown by the  $\chi^2$  values in Table VII Group III.

TABLE VII EFFECT OF MISCELLANEOUS MEDICATIONS

TYPE OF SPECIMEN		M <sub>10</sub>	M	$\chi^2$	CONCLUDED
Group I	Specimens from patients receiving penicillin				
	1	19	063		+ .002
	2	45	086		- .010
	3	161	0.15		0
Group II	Specimens from patients receiving Solu B				
	1	14	0.2		+ .004
	2	174	0.3		+ .00
	3	161	0.32		+ .001
Group III	Normal urine and sulfa compounds				
	Normal urine	---	0.46		+ .003
	Same urine + .5 mg % Na sulfathiazole	---	0.46		+ .00
	Same urine + 25 mg % Na sulfadiazine	---	0.4		+ .00
Group IV	Normal urine and Atabrine				
	Normal urine	194	0.5		+ .002
	Same urine + 1 mg % Atabrine	15	0.55		- .003
	Same urine + 2 mg % Atabrine	244	0.10		- .006
	Same urine + 3 mg % Atabrine	215	0.1		- .008

Range of error expressed as bilirubin - .06 to + .0 mg per cent

$\chi^2$  expected none

This proprietary vitamin B complex for parenteral use contains thiamine, riboflavin, pyridoxine, pantothenate, and nicotinamide.

Atabine (quinacine hydrochloride in amounts of 1, 2, and 3 mg per cent) shows a slight interference as demonstrated in the data of Table VII, Group IV. The 3 mg per cent concentration would mask approximately 0.05 mg per cent of bilirubin, an amount we consider insignificant.

Protein in the urine in amounts greater than 0.1 mg per cent (2 plus ring or acetic acid heat test) causes turbidities which render this method unsuitable. Since most of the commonly used protein precipitants may remove bilirubin with the protein, their use is contraindicated.

In the course of the derivation of the numerical value for  $R_x$ , a few urine specimens (four out of fifty-five) were encountered which gave  $R_x$  values exceeding the mean of 5.19 by about four times the standard deviation. It is safe to assume that these urine specimens contained some interfering material in addition to  $\lambda$ -chromogen. Such an interfering material in an icteric urine would tend to mask a portion of the bilirubin. This effect was observed when two of these urine specimens were used for recovery experiments. In both instances the computed values of  $Y_{\text{orange}}$  were less than expected by an amount corresponding to approximately 0.2 mg per cent of bilirubin. We have been unable to identify the material responsible for this interference.

#### ADAPTATION OF METHOD TO FILTER PHOTOMETER

The method presented here would have limited clinical application if it could not be adapted to filter photometers. Its adaptation to one filter photometer was easily accomplished in our laboratory.\*

Following the exact procedure outlined under Derivation and Application of Correction Equation, a group of ten normal urine specimens and a series of bilirubin solutions ranging from 0.5 to 4 mg per cent were diazotized and read in the Lumetron instrument using orange (580  $m\mu$ ) and blue (420  $m\mu$ ) filters. From these readings the constants  $R_x$  and  $R_y$  were calculated and found to be 8.0 and 0.31, respectively. Substituting these constants in equation (8), it becomes  $(9_a) Y_{\text{orange}} = 1.04 M_{\text{orange}} - 0.13 M_{\text{blue}}$  where the symbols are analogous to those of equation (9) for the Coleman instrument.

TABLE VIII. ADDITION OF BILIRUBIN TO URINE, RECOVERY ON A LUMETRON PHOTOMETER

CASE	$M_{\text{orange}}$ OBSERVED	$M_{\text{blue}}$ OBSERVED	$Y_{\text{orange}}$ COMPUTED	$Y_{\text{orange}}$ ADDED	$Y_{\text{orange}}$ COLUMN 4 MINUS COLUMN 5
38	149	252	122	125	- 003
39	146	215	124	125	- 001
40	149	260	121	125	- 004
41	143	248	117	125	- 008
42	152	337	114	125	- 011
43	260	244	238	252	- 014
44	260	194	245	252	- 007
45	268	306	238	252	- 014
46	264	178	249	252	- 003
47	260	222	241	252	- 011

Mean deviation regardless of sign 0.076

Range of error expressed as bilirubin - 0.08 to -0.11 mg per cent.

Average per cent recovery of added bilirubin 96 per cent.

\*A Lumetron photometer model 400A was employed.

A calibration was carried out using the procedure outlined under Method A. A conversion factor (K) of 8.1 was obtained which was constant up to the 4 mg per cent concentration. Thus 8.1 times  $Y_{\text{orange}}$  equals milligrams per cent bilirubin.

The validity of equation (9a) was tested with mixtures of normal urine and bilirubin as described (Derivation and Application of Correction Equation). The data of this experiment are shown in Table VIII. The recoveries appear to be entirely satisfactory.

As an additional check on the reliability of the method using the filter photometer, a parallel series of determinations were done on icteric urine specimens using both instruments. The results show close agreement, as can be seen in the data of Table IX.

TABLE IX. BILIRUBIN IN ICTERIC URINE DETERMINED SIMULTANEOUSLY ON TWO PHOTOMETERS

19 TO 99 MG %		100 MG % AND OVER	
COLEMAN	LUMETRON	COLEMAN	LUMETRON
19	23	104	115
20	27	111	122
22	28	121	132
22	36	126	139
23	37	143	156
24	39	161	173
25	26	168	175
28	36	169	181
30	30	177	193
35	36	195	200
36	36	201	192
38	28	218	232
38	6	228	228
39	45	228	230
40	52	230	230
41	49	237	254
47	62	240	244
48	67	260	268
52	68	261	260
55	63	272	270
69	79	274	272
70	84	279	284
80	89	294	300
		301	304
		335	320

## DISCUSSION

The method for the determination of bilirubin in urine which we have presented here is essentially a modification or adaptation of the Ehrlich diazo reaction as employed in the Malloy and Evelyn<sup>8</sup> method for serum bilirubin. Since normal or icteric serum has no chromogen other than bilirubin which upon diazotization produces a significant amount of color with a spectral absorption maximum at 530  $m\mu$ , no separation of the bilirubin from the serum is necessary. This makes the determination in serum relatively simple and straightforward.

However, in normal or icteric urine there are amounts of chromogenic material which will react with the diazo reagent to cause a significant inter-

ference with the photometric reading of the diazotized bilirubin. Therefore in analyzing urine for bilirubin by this reaction one is faced with the necessity of (1) separating the bilirubin or its colored derivative from the urine by chemical or physical means, or (2) determining and applying a correction for the interfering material. Attempts at applying the first alternative have so far, to the best of our knowledge, proved unsuccessful. In the present work we have succeeded in employing the second alternative. The correction equation which we have derived was tested by adding known amounts of bilirubin to urine and obtaining satisfactory recoveries. This demonstrated to our satisfaction that neither significant theoretic nor systematic errors were encountered in the procedure.

We believe that we have enhanced the specificity and sensitivity of the determination by converting the red azobilirubin with spectral absorption maximum at  $530\text{ m}\mu$  to the purple azobilirubin with spectral absorption maximum at  $575\text{ m}\mu$  through acidification with hydrochloric acid. This has two beneficial effects. Our data show that in pure solution the red azobilirubin has less optical density at  $530\text{ m}\mu$  than an equivalent amount of purple azobilirubin at  $575\text{ millimicrons}$ . Thus small amounts of bilirubin can be determined more accurately as the purple derivative than as the red derivative. The second beneficial effect is that at  $575\text{ m}\mu$  the interference of the nonbilirubin material which we have designated  $\chi$  chromogen is significantly less than at  $530\text{ millimicrons}$ . Even though the principle on which our correction equation is based would be equally valid for the readings at  $530\text{ m}\mu$ , nevertheless any means of minimizing the effect of the interfering material should be utilized.

With respect to the sensitivity of the method, we estimate a likely error of 0.15 in the computed values of  $Y_{575}$ . This is twice the mean error of our addition and recovery experiments (Table II). Such an error is equivalent to approximately 0.1 mg per cent of bilirubin in urine. In certain instances where two- or threefold dilutions of the urine have been made, the error might be as large as 0.2 to 0.3 mg per cent in terms of bilirubin concentration in the original urine. We feel that this is a liberal estimate of the lower limit of sensitivity of the method. In fact, repeated values of as little as 0.2 mg per hour have been found to date only in patients having disorders of bilirubin metabolism.

We believe that diazotization provides a more specific color reaction for bilirubin than oxidation methods using ferric salts or nitric acid as in the method of Singer and Kubin.<sup>1</sup> The red, blue, and green colored oxidation products are not too well characterized.

Finally, we wish to emphasize the relative simplicity of the procedure. It requires no special apparatus other than a reliable clinical photometer. The required reagents are readily available and easy to prepare. Once the correction equation and the standard curve or calibration factor have been determined for the instrument to be used, the routine performance of the test requires practically no more manipulation than a serum bilirubin determination.



## SUMMARY

A method for the quantitative determination of bilirubin in urine is presented. It employs the principle of direct diazotization of urine with Ehrlich's diazo reagent.

A correction equation is derived and applied for the elimination of nonbilirubin interfering material.

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# A PRACTICAL METHOD FOR THE PREPARATION OF ORGANS FOR THE DETERMINATION OF ANTIBIOTIC CONTENT

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IN THE course of our studies on penicillin tissue levels in small laboratory animals (rats and mice) we were faced with the problem of rapidly emulsifying a large number of organ samples under sterile conditions. The small tissue volumes, their large number, and the time and expense involved precluded the use of the semimicro Waring blender or of mortar and pestle.

We solved this problem by using a Freedom Lighter Duty Flexible Shaft machine (approximately 1/25 horsepower), fitted with a *wire brush*, and 1/2, 1, and 2 ounce jars with metal screw caps (Fig 1). Holes just large enough to accommodate the shaft of the wire brush were made in the covers of the jars. The holes were plugged with cotton and the covered jars were sterilized. The tissues—lung, liver, kidney, and spleen—were removed aseptically and placed in jars appropriate to the volume of the sample. Two volumes of sterile 1 per cent phosphate buffer (pH 6.0) and some sterile aluminum were added. The sterilized wire brushes were inserted through the jar caps and attached to the handpiece. Rotation of the wire brush at approximately 10,000 revolutions per minute produced a finely ground tissue mash in about one minute. This mash was poured into centrifuge tubes and spun at low speed (500 to 1000 revolutions per minute), the clear supernatant liquid was used for assay. Used brushes were washed in 20 per cent NaOH to remove grit and tissue particles, rinsed in tap water, and resterilized.

Eighty to one hundred tissue samples, ranging from 0.5 to 30 Gm each, can be prepared satisfactorily in a single day by this method.

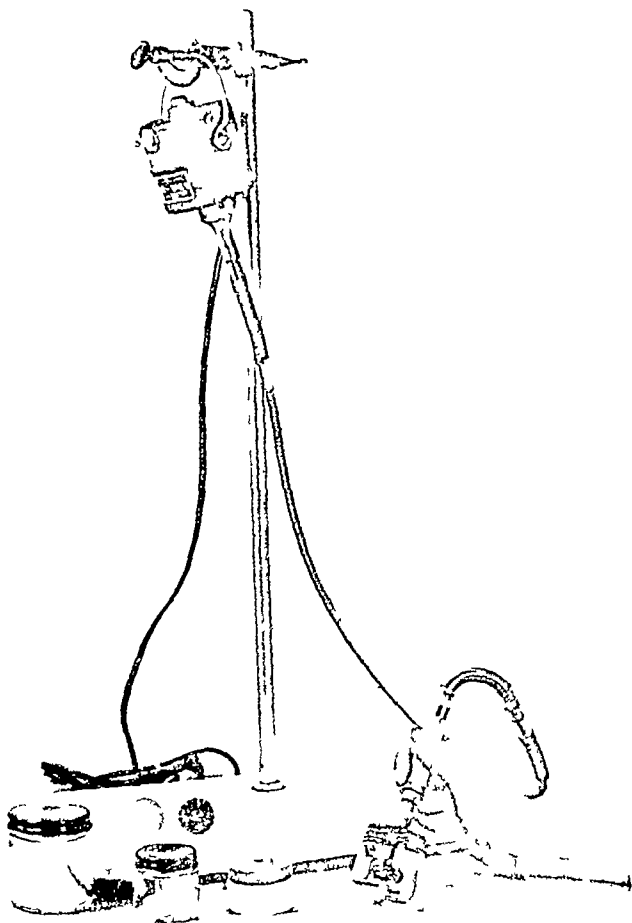


Fig 1

## A RAPID METHOD FOR SERUM CALCIUM DETERMINATION

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IN MANY clinical situations, such as convulsions due to infantile tetany a rapid method for determination of serum calcium, even if somewhat less accurate, would be more valuable to clinicians than the time consuming standard method. A method to be most useful should possess speed and a reasonable degree of accuracy and require a minimum amount of blood.

The method to be described is a nephelometric procedure adapted from the one for determination of calcium in water.<sup>1</sup> Although less precise than a standard micro method, the procedure can attain an accuracy adequate for the usual clinical purposes, it takes less than an hour and requires only 0.1 ml of serum.

Potassium oleate reagent in Duponol solution reacts with calcium ion in ammoniacal solution to give a white colloidal suspension of calcium oleate. The degree of turbidity is proportional to the amount of calcium present in trichloroacetic acid filtrate of serum. The turbidity is measured in a photoelectric colorimeter. The method described is adapted to Klett Summerson (micro) photoelectric colorimeter. Most of the common ions normally present in human serum have no effect on the reaction. Duponol prevents the formation of magnesium oleate, thus eliminating interference due to this ion.

### REAGENTS AND APPARATUS

*Potassium Oleate Reagent*—This reagent is prepared as described by Saifer and Clark.<sup>1</sup> Shake 7.05 Gm of oleic acid\* with a solution of 1.60 Gm of potassium hydroxide in 50 ml of distilled water. Transfer the emulsion by means of 50 ml of 70 per cent ethanol to a flask. Reflux the mixture for one hour and dilute with distilled water to 250 ml in a volumetric flask.

*Duponol Solution*—Prepare 3 per cent solution in distilled water. Duponol P C is an emulsifying reagent †.

*Potassium Oleate-Duponol Reagent*—To each 100 ml of Duponol solution add 20 ml of potassium oleate reagent. Filter off, or remove by centrifugation, any sediment formed. This reagent is stable at room temperature but will come out of solution at lower temperatures. It can be brought back into solution by warming in an incubator at 37° C.

*Twenty per cent Trichloroacetic Acid*—Dissolve 10 Gm of pure trichloroacetic acid in distilled water and make up to 50 milliliters. It is not desirable

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\*Eastman Kodak Company, Rochester, N. Y.

†Sold by Dyestuffs Department of E. I. du Pont de Nemours and Company, Inc., Wilmington, Del.

to make more at a time because the solution is not completely stable. The solution must be kept in the refrigerator when not in use.

*Calcium Standard Solution*—Dissolve 0.5 gm of pure calcite (calcium carbonate) in a 500 ml Lallenmeyer flask with 20 ml of 1 N dilute hydrochloric acid, being careful to avoid spitting. Add about 200 ml of distilled water and boil for a few minutes to drive off the carbon dioxide. Cool to room temperature and transfer quantitatively to a 500 ml volumetric flask. Make up to volume with carbon dioxide free distilled water. Store in a glass stoppered bottle. This standard should be checked for exact calcium content using a permanganate titration procedure. Adjust the concentration of the standard to 40.0 mg of calcium per 100 milliliters.

*Dilute Calcium Standard Solution*—Dilute the preceding standard solution in 100 ml volumetric flasks. Add from a burette 10.0, 15.0, 20.0, 25.0 and 30.0 ml of the calcium standard solution and then add carbon dioxide free distilled water to the mark to get 4, 6, 8, 10 and 12 mg per 100 ml respectively.

*Pipettes*—Two types of pipettes are used in the method: the construction type,<sup>3</sup> modified to a larger volume and syringe pipettes,<sup>4</sup> modified for semiautomatic delivery. The accuracy of a 1 ml syringe is about 0.01 per cent.<sup>5</sup> The syringe pipettes\* are extraordinarily accurate and furthermore they are extremely convenient to handle. When the same volume of reagent is to be added to a number of samples these pipettes will save much time. On the other hand, they are not well suited for pipetting many different samples because the dead space of these pipettes requires a thorough cleaning between each sample. In this case the construction pipettes are more convenient. When the composition of the samples does not differ too much the washing of the pipettes can be neglected. The accuracy of a 1 ml construction pipette is about 0.1 per cent.<sup>6</sup>

The sizes of construction pipettes used are 0.5 ml and 13 milliliters†. With syringe pipettes it is convenient to have pipettes in the sizes of 1 and 20 ml to deliver 0.5 and 10 ml respectively.

#### PROCEDURE

Deliver 0.5 ml of serum into a 12 ml Pyrex centrifuge tube. Add 10 ml of distilled water (with a 2 ml syringe pipette adjusted to deliver 10 ml). Then add 0.5 ml of 20 per cent trichloroacetic acid (with syringe pipette adjusted to deliver this amount). Mix the contents thoroughly with a paraffined wooden applicator and allow it to stand for five minutes. Centrifuge for ten minutes at 2500 revolutions per minute. Transfer 13 ml of centrifugate (with the construction pipette) into a calibrated colorimeter tube. Add 0.2 ml of concentrated ammonium hydroxide‡. Finally add 1 ml of potassium oxalate Duponol reagent (with a syringe pipette). Allow it to stand for fifteen minutes. Read the turbidity in a Klett-Summerson (micro) photoelectric colorimeter.

\*The syringe pipette frame may be obtained from Northon Tool and Instrument Company. The construction pipette may be obtained from Arthur H. Thomas Company, Philadelphia.

†Another 1 ml syringe pipette adjusted to deliver 0.5 ml is recommended as a time saver.

using a 420 (blue) filter \* Set the zero against the distilled (as described in the Klett-Summerson manual) and then proceed. It has been found most expedient to construct the standard curve as a specimen is analyzed in order to keep error at a minimum of dilute standard solutions of 6, 8, 10, and 12 mg of calcium exactly as the serum would be treated. Record the optical density of the unknown the same way,  $D_u$ . Convert the unknown optical density to milligrams of calcium per 100 ml of serum according to the equation

$$\text{Mg Ca per 100 ml of serum} = \frac{D_u}{D_s} \times \text{Mg Ca per 100 ml of standard}$$

#### EXPERIMENTAL

Saifer and Clark have investigated various conditions necessary for oleate formation for the determination of calcium in water. The conditions were studied in this laboratory to insure optimum calcium oleate formation with trichloroacetic acid filtrate.

*Effect of Variation of Potassium Oleate Reagent*—To 13 ml of trichloroacetic acid centrifugate were added 0.5, 1.0, 1.5, and 2.0 ml of potassium oleate-Duponol reagent, respectively. One milliliter was found to be a suitable amount to obtain the maximum optical density.

*Effect of Variation of Ammonium Hydroxide*—To 13 ml of trichloroacetic acid centrifugate were added varying amounts of concentrated ammonia. Then 1.0 ml of potassium oleate-Duponol reagent was added. Optical densities observed remained the same from 50 c mm of concentration up to 320 cubic millimeters. However, there was a decrease in optical densities observed when 640 c mm or more of ammonium hydroxide was added.

*Effect of Variation of Time*—The reaction seems to take place instantaneously. However, it has been found that a slight increase in turbidity takes place from fifteen minutes to thirty minutes; hereafter the turbidity is almost unchanged.

*Precision of the Method*—The results of ten analyses on one sample of human serum are shown in Table I. The average value is 9.6 mg. of calcium per 100 ml., and the maximum deviation from the average is 0.5 mg. per 100 milliliters.

TABLE II DETERMINATION OF ADDED CALCIUM TO POOLED SERUM

SERUM	KRAMER TISDALL (MACROMETHOD) 2.0 ML. (MG. PER 100 ML.)	RECOVERY	NEPHELOMETRIC 0.5 ML. (MG. PER 100 ML.)	RECOVERY
S	10.1		10.4	
S + 3.9	14.1	40	13.8	34
T	10.3		10.7	
T + 3.9	13.8	35	14.1	34
U	9.9		10.3	
U + 3.9	13.6	37	14.1	8
V	10.8		9.6	
V + 3.6	14.1	33	12.9	3

Each determination shown is the average of triplicate.

*The Extent of Recovery*—The average recovery for the three experiments when 3.9 mg. of calcium were added to 100 ml. of serum was 37 for the Kramer-Tisdall method and 35 mg. per 100 ml. of serum for the nephelometric method. In the case of 3.6 mg. per 100 ml. of serum the recoveries were 33 and 33 mg. per 100 ml. for both the Kramer-Tisdall and the nephelometric methods.

*Relationship of Concentration to Optical Density*—Nephelometric measurements are carried out by the same procedure and instruments used for the measurements of substances in solution, that is by comparison against a series of standards. Transmittance measurement particularly when used in instruments equipped with a photoelectric cell is the most sensitive and satisfactory. For the procedure described, it has been found that optical density is directly proportional to concentration and thus resembles optical density for substances in solution. Therefore in this method turbidity measurement is carried out and results are calculated in the same manner as for light absorption.

## SUMMARY

A rapid method for the determination of serum calcium has been presented. It is possible to obtain the results in an hour on a duplicate sample of 0.5 milliliter. The results have been found to be adequate for clinical purposes.

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## CIRCULATION TIME AN ACCURATE INDICATOR FOR OBJECTIVE MEASUREMENT

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MANY methods for measuring the velocity of blood have been proposed in recent years. Despite this, there are few that are simple to perform and dependable with reference to accurate reduplication of measurement.

The response to the particular substance used may be subjective, such as the sensation of heat,<sup>1,3</sup> neuromuscular stimulation,<sup>4,5</sup> or taste,<sup>6</sup> while objective responses may be manifested by their color<sup>8,9</sup> radioactivity,<sup>11</sup> or fluorescence.<sup>11</sup> Obviously any method depending upon the patients' perception is subject to uncontrollable factors which modify the accuracy and consequently the clinical value of the determination. In addition such procedures are not applicable to the unconscious patient.

During the course of clinical investigations of the state of the circulation in surgical patients with the dye T1824, the arrival of the dye in the vessels of the ear was first studied with the Millikan oximeter.<sup>12</sup> The galvanometer deflection with this instrument was not sufficiently rapid for circulation time measurement, nor was the deflection of sufficient magnitude to produce an unmistakable end point. The photometer to be described gives improved response and deflection. Stability is sufficient to permit valid recording of the changing optical density of T1824 over a considerable period of time. Studies of the mixing time of T1824, using this method, will be reported in a later communication.

The principle involved depends upon the measurement of light intensities which have been adequately filtered to insure relative monochromacy. There are three essential components to the apparatus, namely a stable light source and screening filter which transmit light in the vicinity of 6200 Å, a photocell and direct coupled amplifier of high sensitivity and good stability, and an indicating galvanometer or graphic recorder.

### APPARATUS

*Light Source*—The light source is mounted at one end of the ear piece (4, Fig 1) and utilizes a 6 to 8 volt bulb (Mazda 50) operated at 55 volts. In addition to supplying the necessary light, the bulb also furnishes gentle heat which produces vasodilatation with consequent increase in both blood and dye thickness. This is desirable since an increase in dye thickness produces a reduction in monochromatic light intensity. The lamp current is maintained at a constant value by the introduction of the counter-cell (B2, Fig 2) and re-istatance (Fig 2) connected across R7 (Fig 2). Voltage regulation is better than 0.25 per cent. A wet storage cell (D, Fig 1) is used to operate the light.

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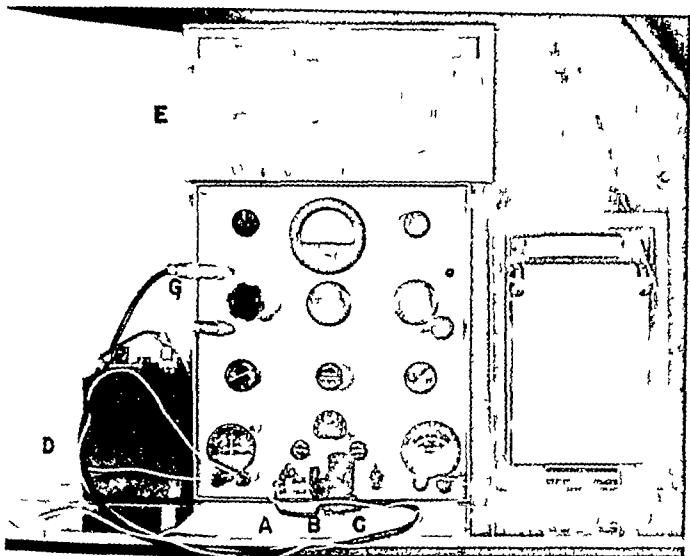


Fig 1—Apparatus for recording circulation time with TIS-11. A Light source B filter compartment C photocell compartment D storage battery for light source E Esterline Angus graphic recording meter G direct coupled amplifier

**Light Filter**—The screening filter is a composite unit consisting of two Corning glasses No 241b and No 9750. The exact spectral transmission is measured with a spectrophotometer lies between 3880Å and 6500Å with 100 per cent transmission at 6100Å and 80 per cent at 6200Å. The filter is placed in the compartment (B Fig 1) in front of the photocell and completely screens all light passing through the ear to the photocell.

**Photocell**—Photronic cells were not considered applicable because of their inherent instability and sensitivity to infrared light.<sup>13</sup> In addition the output of photonic cell is insufficient for recording low values of light intensity with relatively rugged instrument. The photoemissive type of cell RCA No 9-6 was found to be completely suitable because of its spectral response, small size and stability. Adequate light and capacity shielding are provided by means of a small metal cylinder which is securely fastened to the frame of the ear piece (C Fig 1). The light aperture is 1 mm in diameter. The photocell is connected to the amplifier by means of a high insulation shielded cable and coaxial connector. An anode potential of 75 to 10 volts will not produce ionization of the residual gas in the photocell. The response to minute changes in light intensity is instantaneous.

**Amplifier**—In order to obtain maximum sensitivity plus large response from a phototube it is necessary that the grid current of the measuring tube be in the vicinity of 10-12 amperes. Grid current in measuring tubes places a lower limit on the current sensitivity. These characteristics may be obtained in specially designed electrometer tubes or in acorn tubes operated under special conditions. Garbus and Pook<sup>14</sup> and recently Nadel<sup>15</sup> have shown that acorn tubes may be operated with grid current comparable to that

\*Light filter No 60 obtained from Rubicon Company Philadelphia Pa.

<sup>13</sup>Phototubes RCA Manufacturing Company Inc. Camden N J Form PT 001

<sup>14</sup>RP 54 General Electric Company Schenectady N Y D 9647 Western Electric Company Newark N J VIV 41 Victorcen Instrument Company Cleveland Ohio



with a mirror galvanometer movement #3070 and a 12 inch light scale affords unmistakable reading of light intensity. The enclosed light source consists of a penlight type of bulb, a condensing lens, and a 1.5 volt dry battery. The shunt resistance is equal to the critical damping resistance of the movement. An Esterline Angus graphic recorder (Fig 1) with a 1 Ma movement may be used for continuous recording of optical density. Full scale deflection of the recorder is easily obtainable with the standard amount of light passing through an ear of normal thickness and the light filter.

A type RCA #959 acorn tube is used in the first stage. The grid input resistance is selected by means of switch *S5* (Fig 2). Surface leakage in the grid selector switch is reduced to a minimum by first treating the switch with DC 200† and fusing at a temperature of 200 C for at least two hours. The suppressor grid, *G3* and control grid, *G1* are reduced in position. The plate and screen are operated at 6 volts and the filaments at 0.8 volt. These reduced operating conditions improve stability and reduce grid current. A negative bias of 45 volts on the control grid *G3* results in a linear response. The potential of Grid *G1* is maintained at approximately 0.8 volt positive depending upon its effect on the slope and the linearity of the plate curve.

The second and third stage function as voltage and current amplifiers respectively. The output of the acorn tube RCA #959 is fed directly to the grid of an RCA #1N3. A negative bias of 45 volts is sufficient to control plate current. The screen grid and plate are both operated at 45 volts. An RCA #1C3 is used in the last stage with 45 volts applied to the screen and 22.5 volts to the plate. A negative grid bias of 2.5 volts will permit cutoff operations in this stage.

An input resistance of 10<sup>8</sup> ohms will produce full scale deflection of the recording meter when the photocell receives light passing through an ear of normal thickness and the #670 filter. A 1 volt input signal will produce a 0.1 Ma increase in the plate current of the final stage.

The entire amplifier with the exception of the photocell is enclosed in a heavy aluminum cabinet (Fig 1). The tubes and input resistance are firmly supported on the metal panel by means of metal brackets and are further enclosed in an airtight plastic case containing anhydrous calcium chloride. This further reduces atmospheric effects on the surface resistance of the tubes and improves stability. All batteries with the exception of the 6 volt storage cell used for the light source are enclosed in the cabinet. The B batteries are of medium size since the current drain is minimal.

**Operation**—A double pole double throw switch permits the use of the internal galvanometer (Fig 2) together with the variable shunt (*R12* Fig 2) or an external indicating device. The amplifier is placed in operation by means of switch *S1* (Fig 2). A warm up of about five minutes is sufficient to insure stability. The bulb and bucking circuit (*B6* and *R11* Fig 2) are placed in operation by means of *S2* (Fig 2). The bucking circuit is necessary only if the indicating meter goes off scale when using the standard amount of illumination. Zero setting of the indicating galvanometer is determined by the position of *R4* (Fig 2). With the amplifier operating slightly above zero bias the inverse feedback control (*R3* Fig 2) is gradually rotated clockwise to the point where the meter deflection begins to decrease. This position indicates adequate locking of the input and output signals and effective feedback operation. Further adjustment of the inverse feedback and zero controls is not necessary. The condition of all batteries may be determined from the meter (*M1* Fig 2) by rotating switch *S4* (Fig 2).

#### METHOD

The amplifier is placed in operation. No adjustments of zero or feedback controls are necessary. The patient is placed in a recumbent position. The right arm is elevated to the approximate position of the right auricle.

†Rubicon Company  
Liquid Dimethylsilicone DC 00 supplied by the Dow Corning Corporation Midland Mich

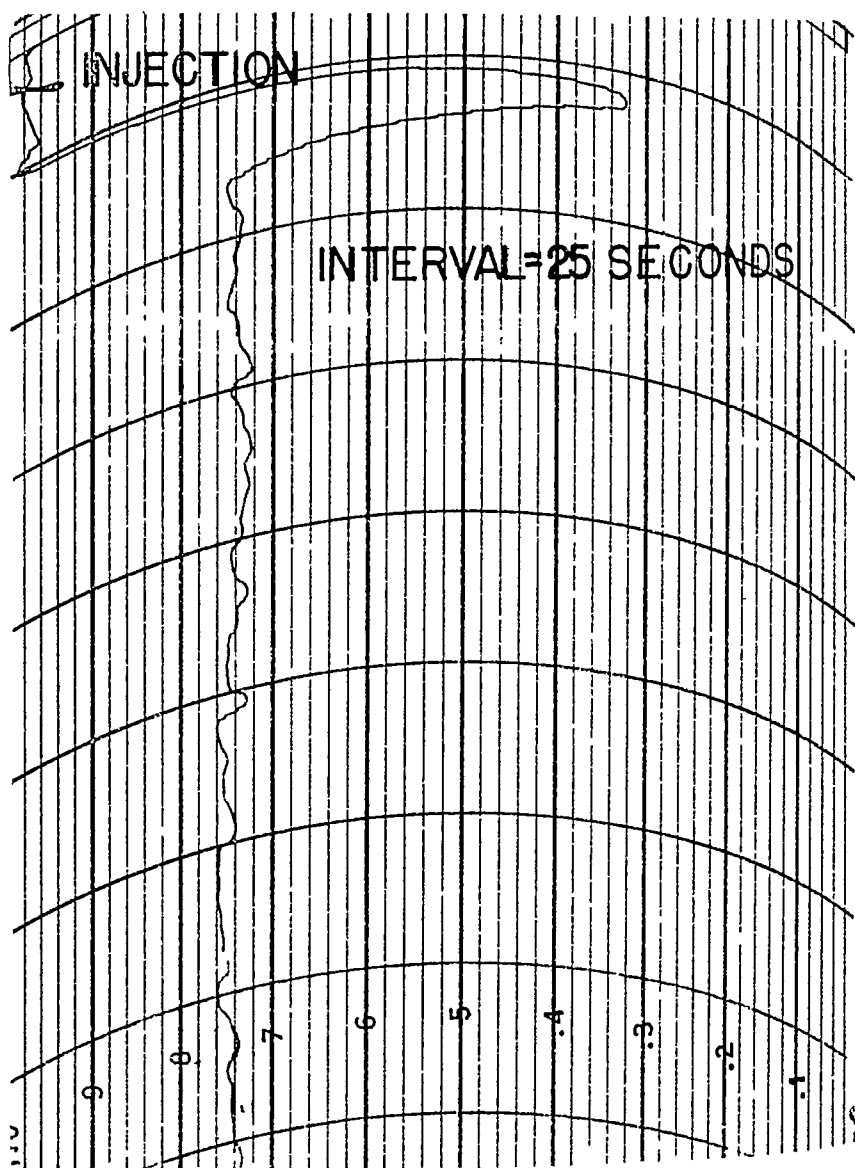


Fig 3—Graphic measurement of arm-to ear circulation time using 20 ml. of T1824

and the ear piece is applied to the right ear. There is no need for warning the patient about any sudden reaction. Illumination is next applied to the ear. The galvanometer deflection will decrease slightly within the next few minutes and then come to rest. This is due to increasing vasodilatation. Full scale deflection is next obtained by means of a potentiometer placed in the output circuit. An 18 gauge needle is then inserted into the antecubital vein of the right arm and after a short pause approximately 3 cc. of the dye\* are

\*0.5 per cent sterile solution of T1824 supplied by William Warner Company N. Y.

rapidly injected. A stop watch may be used to determine the time interval, to the nearest 0.5 second between the moment of injection and the beginning of galvanometer deflection.

Some slight difficulty may be encountered in differentiating the beginning of galvanometer deflection from the steady fluctuation of the galvanometer due to respiration. The error in this regard is of no clinical significance since it is always below 0.5 second. This difficulty is eliminated by using a graphic recorder. If a recording meter is used the time interval may be determined by measuring the time base (Fig. 3). Circulation time measurements may be made to 0.1 second with this method. The determination may be repeated if desired.

#### OBSERVATIONS

A series of circulation time measurements using T1824 was made on a group of eighteen patients who did not exhibit any clinical evidence of congestive heart disease. In addition repeat measurements were made within fifteen minutes using smaller quantities of dye (Table I). These values were then compared with those obtained with a standard clinical procedure using calcium gluconate.

Circulation time measurements with T1824 varied between 10.0 and 21.0 seconds, with an average of 14.2 seconds. In one instance both initial and repeat determinations revealed a circulation time of 21.0 seconds. This was observed in a 68 year old emaciated white male patient. Two sets of determinations were obtained in Negro patients. In these instances the galvanometer deflection was reduced but was still within adequate range to reveal a sharp end point. The greatest difference between repeat measurements was 2.0 seconds (Table I).

Measurement of the circulation time with calcium gluconate varied between 12.0 and 25.0 seconds with an average of 18.0 seconds. In three instances the patients were confused in relation to the moment of heat sensation in the pharynx. The difference between the average circulation time obtained with T1824 and that obtained with calcium gluconate for each individual varied between 0.8 and 6.5 seconds.

#### DISCUSSION

Circulation time measurements obtained with T1824 are in general agreement with those reported for methods using various chemical substances. Goldberg<sup>1</sup> using calcium gluconate reported normal circulation times between 10 and 16 seconds. Bernstein and Simpkins<sup>2</sup> reported an average of 12.9 seconds for circulation times with magnesium sulfate. Magnesium sulfate has the added advantages of producing a sharper end point and being less toxic than calcium gluconate.<sup>18-20</sup> Average circulation times in normal individuals of 20.8 seconds have been reported when papaverine was used,<sup>4</sup> 12.8 seconds with alpha lobeline<sup>5</sup> and 15.6 seconds with fluorescein.<sup>11</sup> Circulation times measured with calcium gluconate were always longer than those obtained with T1824 in this study.



The differences observed between repeat measurements with T1824 were 20 seconds, or less, in all cases. A difference of 20 seconds was observed in two instances the remainder were within one second. Lihenfeld and Berliner<sup>20</sup> reported differences of 3 to 199 per cent in repeat determinations using alpha lobeline, with failure to obtain any reaction in 20 per cent of their patients. With papaverine differences in duplicate determinations have been as great as 3 seconds.<sup>4</sup>

#### SUMMARY

The azo dye T1824 may be used as an objective measure of circulation time. Inherent errors of subjective methods are eliminated and patients are not aware of any discomfort during the process of the test. The test may be performed in individuals having deeply pigmented skins with only slight sacrifice of galvanometer deflection.

The use of monochromatic light and a high sensitivity indicating apparatus permits detection of minute amounts of dye in the blood stream. Validity of circulation time measurements with T1824 is dependent upon the sensitivity and time constant of the amplifier. The amplifier described has a short time constant and high degree of sensitivity. Amplification is sufficient to produce galvanometer deflection as high as 10.4 centimeters. In addition provisions are made for continuous recording of dye concentration in the blood stream.

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TABLE I EXPERIMENTAL PROCEDURE AND ARM TO EAR CIRCULATION TIME MEASUREMENTS USING T1824 AND CALCIUM GLUCONATE

PATIENT	APPLIANCE	FILTER	INITIAL MEASUREMENT			FURTHER MEASUREMENT			DIFFERENCE (SLC)	AVERAGE AGE (SEC)	C <sub>2</sub> GLUCONATE CIRCULATION TIME (SEC)	DIFFERENCE BETWEEN C <sub>2</sub> GLUCONATE & DYE METHODS (SEC)
			DYE INJECTED (MG)	CIRCULATION TIME (SEC)	DEFLATION (CM)	DYE INJECTED (MG)	CIRCULATION TIME (SEC)	DEFLATION (CM)				
A S	Ovimeter	Standard	15	120	1.8							
			15	110	0.8							
			5	130	0.4							
M R	Ovimeter	Standard	20	120	1.0							
			20	130	0.9							
			12	140	0.6							
A R	Ovimeter	Standard	20	150	1.4							
J R	Ovimeter	Standard	20	130	1.6					130		
P W	Photometer	Wratten*29	15	110	127	5	130	0.6		115	140	+23
F H	Photometer	Wratten 25	20	120	2.0	15	115	1.2	1.0	117	175	+18
L D	Photometer	Wratten 29	20	160	2.0	5	155	0.9	0.5	157		
			10	170	1.0	5	165	0.5	0.5	167		
J B (Negro)	Photometer	Wratten 29	20	170	0.7	15	175	0.8	0.5	172	190	+18
L T	Photometer	620	20	100	7.6	4	105	1.5	0.5	103	120 face	+38
										140 tongue		
W M	Photometer	620	20	115	4.1	5	120	2.9	0.5	117	140	+23
L C	Photometer	620	17	210	4.4	8	210	1.9		210	250	+40
R McG	Photometer	620	20	140	5.4	4	140	1.8		140	190	+50
H S	Photometer	620	20	140	5.8	4	145	1.5	0.5	142	180	+38
L T	Photometer	620	20	10	3.6	4	120	0.7	2.0	110	160	+50
(Negro)												
N D	Photometer	620	17	170	1.8	8	170	0.7		170	190	+20
H B	Photometer	620	20	130	6.1	4	140	0.6	1.0	135	200	+65
R S	Photometer	620	17	120	8.1	4	110	3.1	2.0	130	190	+60
T B	Photometer	620	20	170	10.4	4	180	2.1	3.0	175	210	+35
H R	Photometer	620	20	150	9.5	3	152	2.0	0.2	151	210	+59
Average			182	112	5.7	66	117	2.0	0.7	153		

\*Wratten 29 filter is obtained from Kodak Company. Tech. at N.Y. Tech. at this time is not in use with foot film. Average is for 1 hr. 3



# ANTIHYALURONIDASE ANTAGONISTIC TO INFLUENCE OF HYALURONIDASE IN THE SERUM OF NORMAL HUMAN BEINGS AND PATIENTS WITH PNEUMOCOCCIC PNEUMONIA RISE OF TITER IN BACTEREMIC PNEUMOCOCCIC PNEUMONIA

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THE problem of rapidly fatal pneumococcal pneumonia at the Cincinnati General Hospital has prompted the present inquiry into the pathogenesis of this disease. Approximately one fourth of the fatalities due to pneumococcal pneumonia at this hospital in the last three years occurred within twenty four hours of the patient's admission.<sup>1, 2</sup> Between July 1, 1945 and July 1, 1947 there were 426 patients with pneumococcal pneumonia admitted including those with purulent complication on admission the mortality has been 15 per cent.

In 1931, from a study of human autopsy specimens Loeschel<sup>3</sup> concluded that actively advancing pneumococcal pneumonia spread within a lobe of lung by progression of a peripheral zone of edema fluid containing pneumococci from alveolus to alveolus through the pores of Kohn. This was corroborated experimentally by Robertson, Coggeshall and Terrell,<sup>4</sup> Gunn and Nungester and Wood.<sup>5</sup> These investigators found that spreading experimental pneumococcal pneumonia was marked by a peripheral zone of alveoli containing fluid and pneumococci which preceded definite change in the appearance of the alveolar cells or the migration of erythrocytes and polymorphonuclear leucocytes into the alveoli. Robertson and associates<sup>4</sup> also described regurgitation of this infected alveolar fluid through communicating bronchioles into previously uninvolved alveoli of the same lobe of lung. Robertson and Hamburger described the interlobar spread of experimental pneumococcal pneumonia by the intrabronchial flow of infected fluid exudate.

Filtrates of virulent cultures of pneumococcus contain spreading factor (diffusion factor) which produces edema when injected into the flank skin of the rabbit.<sup>6</sup> This edema producing effect of spreading factor was demonstrated to be enzymic when Chain and Duthie<sup>14</sup> established the identity of spreading factor and hyaluronidase in 1939. In a survey of in vitro hyaluronidase production by eighty one strains of pneumococcus isolated from successive cases of pneumonia, Humphrey found no correlation between the amount of hyaluronidase produced and the clinical severity of pneumonia.<sup>17</sup> However the in vitro production of hyaluronidase by pneumococci, which varies according to the availability of hyaluronic acid in the medium and the duration of incubation of the

This work was done in the Departments of Internal Medicine and Biochemistry of the College of Medicine of the University of Cincinnati and the Cincinnati General Hospital.

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culture, may not truly reflect the elaboration of hyaluronidase during pneumococcal infection. Therefore the present investigation was undertaken to study *in vivo* elaboration of hyaluronidase during pneumococcal pneumonia in man.

Spreading factor was first described by Duran-Reynals in 1928<sup>9</sup> when the lesion of vaccinia infection of the rabbit was enlarged by aqueous extracts of rabbit, guinea pig, and rat testicle. The spreading effect was characterized by a zone of edema and was ascribed to an increase of tissue permeability by McClean<sup>10</sup> and by Hoffman and Duran-Reynals.<sup>11</sup> Duran-Reynals found this spreading factor in filtrates of only invasive strains of staphylococci and streptococci and not in noninvasive strains.<sup>12</sup>

Hyaluronidase is the enzyme which hydrolyzes the viscous mucopolysaccharide called hyaluronic acid.<sup>13</sup> Hyaluronic acid, the substrate, has been extracted from mammalian vitreous humor, synovial fluid, umbilical cord,<sup>13</sup> and skin.<sup>14</sup> There is indirect evidence of the presence of hyaluronic acid in lung tissue.<sup>17</sup> Hyaluronic acid is soluble in water and will not dialyze through a porous cellophane membrane. The exact chemical composition of hyaluronic acid is undetermined, but it is known to contain equimolar amounts of hexosamine, acetyl, and glucuronic acid.<sup>15</sup> Hyaluronic acid, the substrate, is not antigenic,<sup>16</sup> in contradistinction to hyaluronidase, the enzyme, which is antigenic.<sup>20</sup> The studies of Bensley<sup>18</sup> suggest that hyaluronic acid is one of the components of the intercellular ground substance of loose connective tissue. Accepting the probability of this premise, Duran-Reynals has proposed the hypothesis that infection by invasive bacteria is induced by hydrolysis of the mucoid ground substance of connective tissue.<sup>19</sup>

Hyaluronidases are antigenic, as demonstrated by McClean in 1943.<sup>21</sup> Therefore it was proposed to estimate the amount of pneumococcus hyaluronidase elaborated in selected cases of pneumococcal pneumonia by titration of the corresponding antihyaluronidase which was elaborated in the serum of each patient. A series of sera was drawn on each patient tested and rises of antihyaluronidase titer in a given patient's series of sera were taken to indicate elaboration of hyaluronidase during that patient's infection.

Three groups of patients, representing three rather definite degrees of severity of primary pneumococcal pneumonia, were selected for these tests. Qualifications of the patients selected for the respective groups were as follows: Negative blood culture and no purulent complication, pneumococcus bacteremia and no purulent complication, and pneumococcus bacteremia with purulent complication. All of these patients were studied while they were under treatment for pneumonia at the Cincinnati General Hospital. All patients included here had blood culture taken on admission to the hospital, and initial serum was drawn within three days of admission.

A preliminary report of this work was published in abstract form in 1946,<sup>21\*</sup> and a later report was published in abstract form in 1947.<sup>22\*</sup>

\*These reports were read at the 1945 and the 1947 Meetings of the Central Society for Clinical Research.

## METHODS

Serial dilutions of the serum to be tested were incubated with a constant amount of hyaluronidase, and then these incubates were tested for residual hyaluronidase activity by Byers' modification<sup>23</sup> of McClean's mucoprotein clot prevention (MCP) test<sup>20</sup>. The highest dilution of the serum which inactivated a constant amount of hyaluronidase was taken as the antihyaluronidase titer of that serum.

The hyaluronidases used in these tests were Seitz filtered seventy two hour cultures of mouse virulent Types 1, 2 and 7 pneumococci in fluid medium which contained 0.75 per cent potassium hyaluronate and no glucose. The potassium hyaluronate used was prepared by the method of Byers, Tytell and Logan.<sup>24</sup> The three hyaluronidases used in the preliminary tests<sup>21</sup> were prepared by Gibert in the semisynthetic medium which she developed for pneumococci.<sup>25</sup> The three hyaluronidases used in subsequent tests were prepared in beef heart infusion broth (Difco). By the mucoprotein clot prevention test five of these hyaluronidases were active in the dilution of at least 1:16,000, and the sixth was active in the dilution of 1:1,200.

The constant amount of hyaluronidase used throughout these tests was 0.25 cc of the unrefined hyaluronidase which had been diluted so that a 1:8 or a 1:16 dilution prevented the mucoprotein clot, regardless of the original potency of the preparation used. This dilution of each hyaluronidase was freshly prepared each day and was used to supply all tests done that day. All dilutions of hyaluronidase and of serum to be tested were made in 1 per cent proteose peptone in physiologic saline. This solution minimized a partial loss of potency which occurred when the hyaluronidases were diluted in distilled water or physiologic saline.

All of the sera in one patient's series of sera were tested at one time with one hyaluronidase preparation. Five to seven normal sera and the serum of Patient A. T. \* as the control were tested at one time with the Type 2 pneumococcus hyaluronidase. Twofold serial dilution beginning with 1:2 was made for each serum tested. Usually ten dilutions were made for each serum, but more were added as needed to obtain an end point. Serum dilutions were made in the volume of 0.5 cc in tubes 100 by 13 millimeters. The constant amount of hyaluronidase in the volume of 0.25 cc was added to each serum dilution and to a control tube containing 0.5 cc of proteose peptone saline with no serum. The tubes were mixed by shaking the tube rack then they were incubated at room temperature (25°C) for twenty minutes. The final dilution of serum in the first tube was 1:3 and the final volume in each tube was 0.75 cubic centimeter.

Residual hyaluronidase activity which remained after incubation of the serum dilution and hyaluronidase mixtures was determined as follows.

(1) To each tube was added 1.0 cc of substrate. This substrate contained equal volumes of three components: dialyzed tryptic digest of umbilical cord

\* This patient was six months convalescent from Type 2 pneumococcus tricuspid endocarditis with repeated septic pulmonary emboli which were complicated by Type 2 pneumococcus empyema.

which contained approximately 0.20 per cent hyaluronic acid,<sup>21</sup> normal horse serum which exhibited neither hyaluronidase nor antihyaluronidase activity, and distilled water.

(2) The tubes were mixed by shaking the tube rack. They were incubated in a water bath at 37° C. for thirty minutes, and then cooled in an ice water bath for five minutes.

(3) To each tube was added 1.0 cc. of 5 per cent acetic acid, and each tube was shaken individually and read for formation of the mucoprotein clot. The highest dilution of a given serum which exhibited either heavy or thready clot was taken as the antihyaluronidase titer of that serum. Results were expressed in terms of dilution of serum prior to addition of the substrate. Activity of the constant amount of hyaluronidase was demonstrated in the control tube by prevention of the clot.

The mucoprotein clot prevention had a standard error of about 22 per cent as estimated by McClean in his own use of the test.<sup>20</sup> The determination of antihyaluronidase, as done here, permitted a twofold chance variation of titer of a given serum, but fourfold variation did not occur. For example, antihyaluronidase determinations were repeated eight times on the serum of Patient A T in its use as a control serum, with titers of 1:384 six times and titers of 1:192 two times.

TABLE I ANTIHYALURONIDASE ANTAGONISTIC TO PNEUMOCOCCUS HYALURONIDASE (FROM TYPE 2 PNEUMOCOCCUS), SERUM TITERS OF NORMAL HUMAN BEINGS COMPARED WITH THE SERUM TITERS OF CONVALESCENT PATIENT A T\*

NO	TITER†		NORMAL A T	NO	TITER†		NORMAL A T
	NORMAL SUBJECT	PATIENT A T			NORMAL SUBJECT	PATIENT A T	
1	1:192	1:192	1	26	1:48	1:384	1:8
2	1:192	1:192	1	27	1:48	1:384	1:8
3	1:192	1:384	1:2	28	1:48	1:384	1:8
4	1:192	1:384	1:2	29	1:48	1:384	1:8
5	1:96	1:192	1:2	30	1:48	1:384	1:8
6	1:96	1:192	1:2	31	1:24	1:192	1:8
7	1:96	1:384	1:4	32	1:24	1:192	1:16
8	1:96	1:384	1:4	33	1:24	1:384	1:16
9	1:96	1:384	1:4	34	1:24	1:384	1:16
10	1:96	1:384	1:4	35	1:24	1:384	1:16
11	1:96	1:384	1:4	36	1:24	1:384	1:16
12	1:96	1:384	1:4	37	1:24	1:384	1:16
13	1:96	1:384	1:4	38	1:24	1:384	1:16
14	1:48	1:192	1:4	39	1:12	1:196	1:32
15	1:48	1:192	1:4	40	1:12	1:384	1:32
16	1:48	1:192	1:4	41	1:12	1:384	1:32
17	1:48	1:384	1:8	42	1:12	1:384	1:32
18	1:48	1:384	1:8	43	1:12	1:384	1:32
19	1:48	1:384	1:8	44	1:12	1:384	1:32
20	1:48	1:384	1:8	45	1:12	1:384	1:32
21	1:48	1:384	1:8	46	1:12	1:384	1:32
22	1:48	1:384	1:8	47	1:12	1:384	1:32
23	1:48	1:384	1:8	48	1:6	1:192	1:64
24	1:48	1:384	1:8	49	1:6	1:384	1:64
25	1:48	1:384	1:8	50	1:6	1:384	

\*This patient was six months convalescent from Type 2 pneumococcus triculpid pneumonia and multiple septic pulmonary emboli with Type 2 pneumococcus pneumonia.

†The highest dilution of the serum which inactivated the constant amount of hyaluronidase used in the test.

## RESULTS

*Normal Human Serum*—Antihyaluronidase antagonistic to Type 2 pneumococcus hyaluronidase was present in the serum of two normal human beings in the same titer as that of Patient A. T four were  $\frac{1}{2}$  the titer of Patient A. T ten were  $\frac{1}{4}$ , sixteen were  $\frac{1}{8}$ , seven were  $\frac{1}{16}$ , nine were  $\frac{1}{32}$  and two were  $\frac{1}{64}$  (Table I).

*Serum of Pneumococcic Pneumonia Patients*—Serial sera of twenty six patients with pneumonia were tested seven patients had negative blood culture and no purulent complication, eleven patients had pneumococcus bacteremia and no purulent complication eight patients had pneumococcus bacteremia with purulent complications.

TABLE II. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA. BLOOD CULTURE NEGATIVE AND NO PURULENT COMPLICATIONS. TABULATION OF SERUM ANTIHYALURONIDASE TITERS PLOTTED ON THE GRAPHS IN FIG. 1

PN TYPE	DAYS ILL BEFORE ADMISSION	DAYS FROM ADMISSION TO FIRST SERUM	DAYS FROM FIRST SERUM TO HIGHEST TITER	TYPE OF PN HYALURONIDASE ANTAGONIZED	TITER OF FIRST SERUM	TITER OF HIGHEST TITER
3	7	0	18	1	12	12
			18	2	12	12
18	10	1	21	1	24	48
			21	2	24	48
1	3	0	32	7	48	96
7	1	0	19	2	384	384
			19	7	192	192
			5	1	192	384
20	Unknown	3	14	2	96	48*
			5	7	96	192
7	4	0	8	1	24	6
			8*	2	24	12
6†	2	1	10	1	384	192
			10	2	192	192
			10	7	96	48

Antihyaluronidase titers decreased

†This patient died on the fifteenth hospital day of left ventricular cardiac failure

*Serum of Patients With Negative Blood Culture and no Purulent Complication*—Antihyaluronidase responses obtained in the seven patients in this group are presented in Table II and Fig. 1 and the sequence of cases in Table II corresponds with the sequence of graphs in Fig. 1. The sera of two patients were titrated against Type 1, Type 2 and Type 7 pneumococcus hyaluronidases, sera of four patients were titrated against two of these hyaluronidases and sera of one patient were titrated against only one hyaluronidase. Changes of antihyaluronidase titer found in these fifteen titrations of serial sera were one fourth fold once, one half fold four times, unchanged five times, and two fold five times. The abscissa of each graph represents the time in days subsequent to hospital admission at which serum samples were drawn from the patient. The ordinate represents the antihyaluronidase titers of the sera tested expressed as the reciprocal of the highest dilutions of sera which inactivated the constant amount of hyaluronidase. Each curve represents the antihyal

uronidase response as titrated against one of the hyaluronidases. From the graphs in Fig 1 it can be seen that none of these patients exhibited rise of serum antihyaluronidase titer which exceeded twofold, representing no rise within the accuracy of the test.

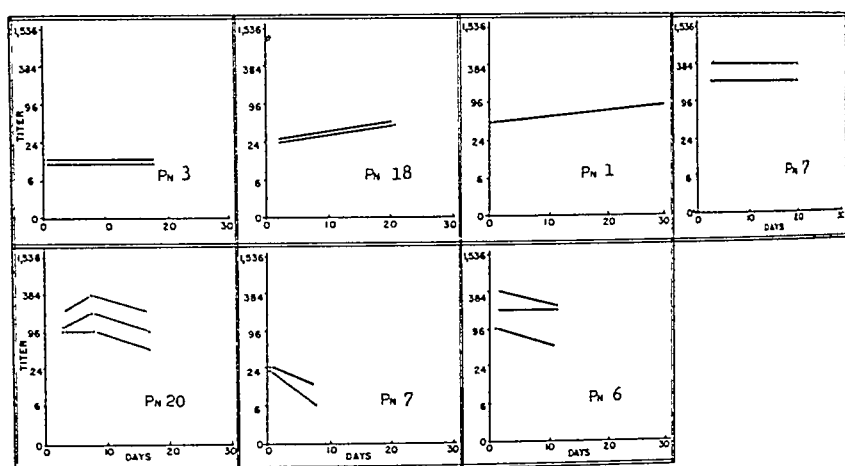


Fig 1—Antihyaluronidase in pneumococcal pneumonia blood culture negative

The clinical data pertaining to these seven patients are shown in Table III. Their average age was 45.7 years. An average of 1.7 lobes was involved by pneumonia,\* and the average hospital stay was 16.4 days. The only death occurred in a 72-year-old woman who died of left ventricular cardiac failure on her fifteenth hospital day, four days after roentgenogram had shown marked resolution of the pneumonia.

TABLE III ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA, BLOOD CULTURE NEGATIVE AND NO PURULENT COMPLICATIONS, CLINICAL DATA

PN TYPE	AGE OF PATIENT (YR)	LOBES OF LUNG INVOLVED	TREATMENT*			COMPLICATIONS	DAYS IN HOSPITAL
			SULFA	PENICILLIN	SERUM		
3	74	LU, RL	+	0	0	0	29
18	29	RU	+	0	0	0	17
1	33	RL	+	0	0	0	11
7	32	LL, RL	+	0	+	0	13
20	54	LL, RU, RL	+	+	0	0	23
7	26	LL	+	0	0	0	7
6†	72	LL, RL	+	+	0	0	15
Average	45.7	1.71					16.4

\*Sulpha. Ordinary doses of sulfamerazine<sup>1</sup> or sulfadiazine units of amorphous penicillin every three hours intramuscularly. Penicillin 20,000 to 25,000 units of antipneumococcal rabbit serum (Lederle). Serum type specific therapy.

†This patient died on the fifteenth hospital day of left ventricular cardiac failure.

*Serum of Patients With Pneumococcus Bacteremia and no Purulent Complication* Antihyaluronidase responses obtained in the eleven patients in this group are presented in Table IV and Fig 2, and the sequence of cases in Table

\*No distinction was made in this or the other groups of patients as to whether the type of pneumonia was lobar or bronchial. The type of infecting pneumococcus was identified in all cases and the distribution of the pneumonia was identified by roentgenogram in all cases.

IV corresponds with the sequence of graphs in Fig 2. The sera were titrated against Type 1, Type 2, and Type 7 pneumococcus hyaluronidases. Rises of antihyaluronidase titer found in these thirty three titrations of serum sera were no change once, twofold seven times fourfold six times eightfold eleven times, and sixteen fold eight times. From the curves in Fig 2 it can be seen that the first eight patients exhibited rises of titer which were fourfold or greater and that six of these eight patients exhibited rises which were eightfold or greater. These increases of antihyaluronidase titer were all apparent during the patients' first week of hospitalization and were not specific for pneumococcus type. In four patients who were observed for more than thirty days the highest rise of serum antihyaluronidase titer occurred thirteen to thirty days after pneumococcus bacteremia. The last three patients in this group exhibited rises of titer which were twofold or less representing no rise within the accuracy of the test.

TABLE IV. ANTIHIALURONIDASE IN PNEUMOCOCCIC PNEUMONIA. PNEUMOCOCCUS BACTEREMIA WITHOUT PURULENT COMPLICATIONS. TABULATION OF SERUM ANTIHIALURONIDASE TITERS PLOTTED ON GRAPHS IN FIG 2.

PN TYPE	DAYS ILL BEFORE ADMISSION	DAYS FROM ADMISSION TO FIRST SERUM	DAYS FROM FIRST SERUM TO HIGHEST TITER	TYPE OF PN HIALURONIDASE ANTAGONIZED	TITER OF FIRST SERUM	TITER OF HIGHEST
7	5	1	12	1	24	96
			12	2	12	96
			12	7	24	192
2	4	2	21	1	48	768
			21	2	48	384
			21	7	48	384
3	Unknown	1	13	1	96	1536
			13	2	192	768
			13	7	96	768
7	5	1	14	1	24	384
			28	2	12	192
			28	7	24	192
10	1	1	17	1	96	1536
			30	2	96	768
			30	7	48	384
14	Unknown	3	15	1	96	768
			29	2	48	768
			29	7	48	768
7	3	2	18	1	96	768
			18	2	96	768
			18	7	24	384
1	3	2	21	1	48	192
			21	2	96	384
			21	7	48	192
1	0	1	6	1	96	192
			6	2	96	192
			6	7	48	192
25	Unknown	1	18	1	96	192
			18	2	48	96
			18	7	24	48
8	5	2	14	1	96	192
			14	2	96	96
			14	7	96	192

All of these patients recovered.

The clinical data pertaining to these eleven patients are shown in Table V. The average age was 45.2 years. An average of 1.6 lobes was involved by pneumonia, and the average hospital stay was 29.0 days. All of these eleven patients recovered.

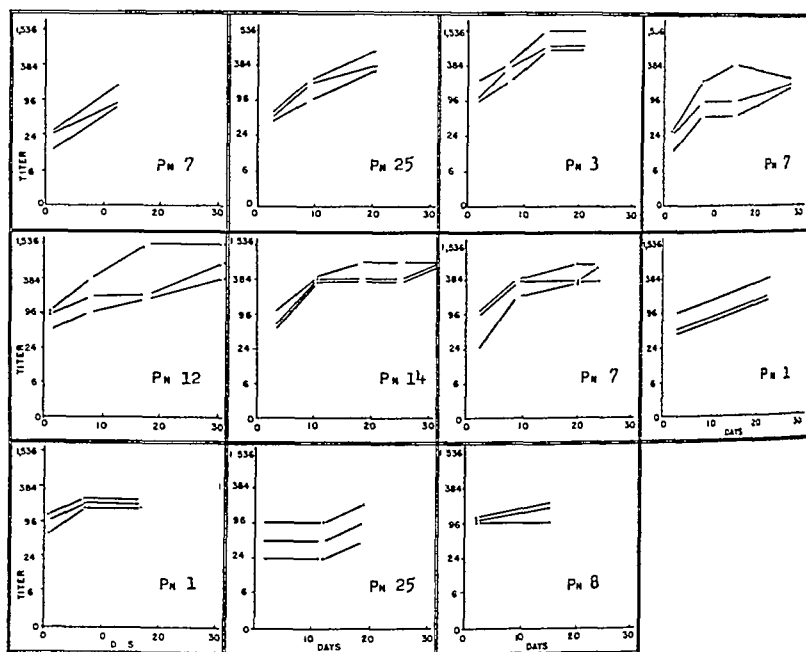


Fig. 2—Antihyaluronidase in pneumococcal pneumonia, pneumococcus bacteremia without purulent complications.

TABLE V. ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA, PNEUMOCOCCUS BACTEREMIA WITHOUT PURULENT COMPLICATIONS, CLINICAL DATA

PN TYPE*	AGE OF PATIENT (YR)	LOBES OF LUNG INVOLVED	TREATMENT†			COMPLICATIONS	DAYS IN HOSPITAL
			SULFA	PENICILLIN	SERUM		
7	37	RU, RM, RL	+	+	+	Cerebral thrombosis‡	40
25	42	RL	+	+	0	Azotemia	37
3	45	LL, RU	+	0	0	Azotemia	24
7	58	RU, RM	+	+	0	Pleural effusion	60
12	35	RU	+	+	0	0	10
14	58	RM, RL	+	+	0	Pulmonary infarction‡	40
7	44	RL	+	+	0	Delayed resolution	28
1	49	RM, RL	+	0	0	0	9
1	18	LL	+	+	0	0	19
25	65	LU, LL	+	+	0	Azotemia	30
8	47	RU	+	0	0	0	1
Average	45.2	1.63					29.0

\*All of these patients recovered.

†Sulfa: Ordinary doses of sulfamerazine<sup>1</sup> or sulfadiazine of amorphous penicillin every three hours intramuscularly antipneumococcal rabbit serum (Lederle).

penicillin 20,000 to 200,000 units serum type specific therapy.

‡These complications occurred during convalescence.



*Serum of Patients With Pneumococcus Bacteremia With Purulent Complications* Antihyaluronidase responses obtained in the eight patients in this group are presented in Table VI and Fig. 3 and the sequence of cases in Table VI corresponds with the sequence of graphs in Fig. 3. The sera were titrated against Type 1, Type 2, and Type 7 pneumococcus hyaluronidases. Rises of antihyaluronidase titer found in these twenty four titrations of serial sera were fourfold six times eightfold five times sixteen fold three times thirty two fold

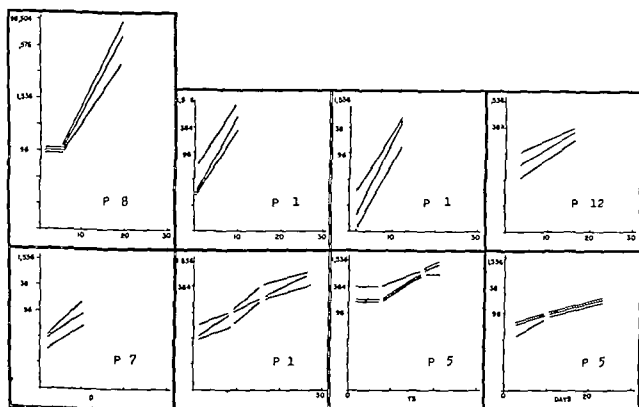


Fig. 3—Antihyaluronidase in pneumococcal pneumonia pneumococcus bacteremia with purulent complications

three times, and sixty four fold or greater seven times. From the curves in Fig. 3 it can be seen that all eight patients exhibited rises of titer which were four fold or greater, and that four patients exhibited increases which were eightfold or greater. Rises of antihyaluronidase titer in six patients were apparent during the first week of hospitalization and in all patients were not specific for type of pneumococcus. In five patients who were observed for more than thirty days the highest rise of serum antihyaluronidase titer occurred ten to twenty seven days after pneumococcus bacteremia.

The clinical data pertaining to these eight patients are shown in Table VII. The average age was 48.3 years. An average of 1.7 lobes was involved by pneumonia, and the average hospital stay was 41.0 days. The first six patients recovered, the seventh died of empyema on the twenty fifth hospital day and the eighth died of multiple lung abscesses on the thirty second hospital day.

*Particular Considerations of the Antihyaluronidase in the Serum of Patients With Pneumococcal Pneumonia* Studies which indicate that the antihyaluronidase in these sera was specifically antagonistic to hyaluronidase elaborated by the species pneumococcus have been reported elsewhere.<sup>9</sup>

TABLE VI ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA, PNEUMOCOCCUS BACT. PNE.  
WITH PURULENT COMPLICATIONS, TABULATION OF SERUM ANTIHYALURONIDASE TITERS  
PLOTTED ON THE GRAPHS IN FIG 3

PN TYPE	DAYS ILL BEFORE ADMISSION	DAYS FROM ADMISSION TO FIRST SERUM	DAYS FROM FIRST SERUM TO HIGHEST TITER	TYPE OF PN HYALURONIDASE ANTAGONIZED	TITER OF FIRST SERUM	TITER OF HIGHEST TITER
8	3	1	19 48 48	1 2 7	96 96 96	4915 19000 24000
1	4	0	8 31 8	1 2 7	48 12 12	1000 150 300
1	4	1	12 12 12	1 2 7	12 3 <3	100 100 100
12	9	3	47 89 14	1 2 7	96 48 24	300 300 100
7	Unknown	1	10 10 10	1 2 7	12 24 24	40 90 100
1	Unknown	1	26 26 26	1 2 7	48 24 24	700 100 100
5*	3	1	19 16 19	1 2 7	384 192 192	1000 100 1000
5†	7	2	22 22 22	1 2 7	48 24 48	100 100 100

\*This patient died of empyema

†This patient died of multiple lung abscesses

TABLE VII ANTIHYALURONIDASE IN PNEUMOCOCCIC PNEUMONIA, PNEUMOCOCCUS  
BACTEREMIA WITH PURULENT COMPLICATIONS, CLINICAL DATA

PN TYPE	AGE OF PATIENT (YR)	LOBES OF LUNG INVOLVED	TREATMENT*			COMPLICATIONS	DAYS IN HOSPITAL
			SULFA	PENI CILLIN	SERUM		
8	17	RL	+	0	0	Empyema	61
1	37	LL	+	+	+	Empyema	100
1	43	LL	+	0†	0	Empyema	63
12	71	RU	+	+	0	Pyarthrosis, azotemia	41
7	52	RU, RM, RL	+	+	+	Meningitis	61
1	52	RU, RM, RL	+	+	0	Empyema	20
5†	55	RL	+	+	0	Empyema	2
5†	60	LL, RU, RL	+	+	0	Lung abscess	110
Average 48.3		175					

\*Sulfa, Ordinary doses of sulfamerazine<sup>†</sup> or sulfadiazine penicillin 20 000 to 20 000 units of amorphous penicillin every three hours intramuscularly serum type specific therapy antipneumococcal rabbit serum (Lederle)

†This patient received intrapleural penicillin only

‡These patients died on the twenty-fifth and thirty-second hospital day respectively

Therapeutic antipneumococcal rabbit serum exhibited very little anti hyaluronidase. The highest dilution of Type 2 concentrated antipneumococcal serum\* which antagonized the constant amount of Type 2 pneumococcus hyaluronidase was 1:12.

\*The antisera tested were Lederle Lot No 472H676J 50 000 units per vial, and Lot No B4555 20 000 units per vial

Intravenous administration of 200,000 units of Type 1 therapeutic anti pneumococcal rabbit serum (Lederle) in two injections to a patient with Type 1 pneumonia did not bring about an increase of the patient's serum antihyaluronidase titer in the two day period following the antiserum but did produce a marked rise in titer of agglutinin for the specific pneumococcus. There were no agglutinins for Type 1 pneumococcus before the antiserum but there was a progressive rise of agglutinin titer to 1:2560 (2 plus) as observed at four intervals in the two day period. One patient with Type 7 pneumonia who was given 200,000 units of Type 7 antipneumococcal rabbit serum intravenously in one injection exhibited no change of serum antihyaluronidase titer twenty days later.

High therapeutic concentrations of penicillin and sodium sulfadiazine did not exhibit antihyaluronidase activity when titrated against pneumococcus hyaluronidase. The concentrations tested were 4 units of penicillin per cubic centimeter (Food and Drug Administration Standard) and 20 mg per 100 cc of sodium sulfadiazine.

Low titer and high titer antihyaluronidase sera from one patient and sera from two other patients exhibited no loss of antihyaluronidase activity following one hour at 56° C in a water bath.

Patients with other pneumococcus infections without pneumonia exhibited rises of serum antihyaluronidase titer. One patient with pneumococcal meningitis secondary to mastoiditis and another patient with mesothelioma of the pleura complicated by pneumococcal empyema exhibited respectively fourfold and eightfold rises of serum antihyaluronidase titer as tested with the three pneumococcus hyaluronidases. One patient with Type 12 pneumococcal aortic endocarditis and no evidence of pneumonia exhibited a rise of serum antihyaluronidase titer from the titer of 1:3 to the titer of 1:192 as titrated with Type 2 pneumococcus hyaluronidase.

#### DISCUSSION

The rises of serum antihyaluronidase titer reported here indicate that hyaluronidase is elaborated in the bacteremic pneumococcal pneumonias. In general following an optimal injection of an antigen there is a lag of a few days before the appearance in the blood of the corresponding antibody. The titer of this antibody rises rapidly to a maximum which usually is attained between the tenth and twenty second day after inoculation<sup>28</sup>. According to these principles elaboration of hyaluronidase in fourteen of sixteen cases of bacteremic pneumococcal pneumonia began during or before the first week of hospitalization.

The relation of this elaboration of hyaluronidase to the severity of pneumococcal pneumonia is not clear. The rises of serum antihyaluronidase titer which were exhibited by all patients with purulent complication suggest that hyaluronidase may promote increased permeability of pleural, meningeal, or synovial tissues. In corroboration of this impression all patients whose serum exhibited no increase of antihyaluronidase titer were free of purulent complica-

tion Increase of antihyaluronidase titer could not be correlated with the number of lobes involved by the pneumonia since the average number of lobes involved was similar in all groups

The antihyaluronidase reported here may be similar to the nonantibacterial therapeutic factor which was reported to be present in antipneumococcal horse serum by Sabin in 1932<sup>27</sup>

The occurrence of small amounts of antihyaluronidase antagonistic to pneumococcus hyaluronidase in the serum of all of fifty normal human beings is notable, but its significance is obscure Ten of these people were high school youths 17 and 18 years of age, and forty were medical personnel of the Cincinnati General Hospital between 20 and 30 years of age

The absence of type specificity of the antihyaluronidase response in pneumococcal pneumonia is consistent with the finding of Frison and Wenner<sup>28</sup> that an inhibitory substance in the serum of rabbits immunized with pneumococcal neutralized enzymes (hyaluronidases) derived from six different types of pneumococci

#### SUMMARY

Antihyaluronidase in the serum of fifty normal human beings antagonistic to Type 2 pneumococcus hyaluronidase was compared with the same antihyaluronidase in the serum of Patient A. T. who was six months convalescent from Type 2 pneumococcus endocarditis All of the fifty normal subjects tested had some degree of this antihyaluronidase Since subsequent experiments demonstrated that serum antihyaluronidase antagonistic to pneumococcus hyaluronidase is not type specific, these observations upon the sera of normal human beings pertain to hyaluronidase elaborated by all types of pneumococci

Serial sera of twenty six patients with primary pneumococcal pneumonia were tested for antihyaluronidase by titration against hyaluronidases in culture filtrates of Type 1, Type 2, and Type 7 pneumococci Seven patients with negative blood culture and no purulent complication exhibited no change of antihyaluronidase titer Eight of eleven patients with pneumococcus bacteremia and no purulent complication exhibited rises of antihyaluronidase titer which were fourfold or greater All of eight patients with pneumococcus bacteremia and purulent complication exhibited rises of titer of fourfold or greater The rises of antihyaluronidase titer which were observed were not specific for pneumococcus type

Therapeutic antipneumococcal rabbit serum exhibited less antagonism to pneumococcus hyaluronidase than did a majority of the sera from fifty normal human beings High therapeutic concentrations of penicillin and sodium sulfadiazine exhibited no antagonism of pneumococcus hyaluronidase

A rise of serum antihyaluronidase titer could not be brought about in one patient with pneumococcal pneumonia by the intravenous administration of therapeutic antipneumococcal serum, but a progressive rise of serum agglutinating titer for the homologous pneumococcus was produced

# CONCLUSION

Rises in the titer of serum antihyaluronidase antagonistic to pneumococcus hyaluronidase, found in sixteen patients with bacteremic pneumococcal pneumonia, indicate that hyaluronidase was elaborated and was antigenic in these pneumococcus infections. This elaboration of hyaluronidase was apparent during the first week of hospitalization in fourteen of these sixteen patients with pneumonia. The elaboration of hyaluronidase appears to be related in some way to the development of purulent complications in pneumococcal pneumonia.

The author wishes to acknowledge the interest and assistance of Dr. M. A. Blinkenhorn, Dr. M. A. Logan, and Dr. A. A. Tytell which were indispensable to the completion of this work.

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## THE ROLL OF HIGH BLOOD PENICILLIN LEVELS ACHIEVED WITH CARONAMID IN PENETRATING THE BLOOD BRAIN BARRIER

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**I**N A previous report<sup>1</sup> we described a method for producing sustained high penicillin levels in the blood. This consisted of frequent rapid intravenous injections of large doses of crystalline penicillin in conjunction with the oral administration of Caronamide (4-carboxyphenylmethanesulfonamide). The latter inhibits tubular excretion of the antibiotic. The method was found to be safe and effective.

In a review of previous investigations, Wile<sup>2</sup> reported disagreement on the question of whether any penicillin traverses the blood brain barrier under normal and abnormal conditions. When penetration into the spinal fluid was reported the levels recorded were low. Smith and collaborators<sup>3</sup> gave 100,000 units of penicillin intravenously to patients with normal and inflamed meninges and found only traces of penicillin in the spinal fluid. They concluded that the meninges are relatively impermeable to penicillin. Schwemlein and co-workers<sup>4</sup> administered penicillin by intravenous drip in amounts varying from 10 to 20 million units in twenty-four hours and found the highest spinal fluid level thus achieved to be 0.55 Oxford unit per milliliter. Because of these and similar findings by other authors, cerebrospinal infections have been treated by the intrathecal route a procedure not without hazard.<sup>5,6</sup>

For the study reported below twelve patients free of infection of the central nervous system were selected. In eight of these twelve patients one million units of crystalline penicillin were administered rapidly intravenously every hour for ten hours. In four, namely Patients 3, 4, 5, and 7, a continuous intravenous infusion of 10 per cent glucose in 1,000 cc of distilled water was administered during the ten-hour period and the penicillin was injected every hour into the distal end of the rubber tubing. Eight patients received Caronamide in doses of 4 Gm. every three hours starting eighteen hours prior to and continuing during the period of administration of the antibiotic. These included six patients who received the hourly injections and two who received penicillin along with a continuous intravenous injection of glucose. For comparison Caronamide was withheld from the remaining four patients.

The peripheral venous blood and the spinal fluid samples were obtained approximately fifteen minutes after the last (tenth) injection of the penicillin. In some of the patients specimens also were obtained fifteen minutes after the fifth as well as the tenth injection and fourteen hours after the last (tenth) injection.

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The method of penicillin assay was a broth tube dilution method using *Staphylococcus aureus*, strain H, as the standard organism and fresh meat extract broth as the medium. The minimal concentration of the standard penicillin\* required to inhibit the inoculum of  $5 \times 10^2$  *Staph aureus* strain H cells was 0.02 unit per milliliter. All titrations of blood and spinal fluid levels were accompanied by and compared with this standard.

TABLE I. PENICILLIN LEVELS IN THE BLOOD AND CEREBROSPINAL FLUID AFTER THE ADMINISTRATION OF ONE MILLION UNITS OF CRYSTALLINE PENICILLIN EVERY HOUR FOR TEN HOURS WITH AND WITHOUT CARONAMIDE (IN OXFORD UNITS PER MILLILITER)

PATIENT	TIME					
	AFTER FIFTH INJECTION		AFTER TENTH INJECTION		14 HOURS LATER	
	BLOOD	SPINAL FLUID	BLOOD	SPINAL FLUID	BLOOD	SPINAL FLUID
<i>With Caronamide</i>						
1	266.0	2.0	160.0	4.4	2.0	0.8
2	260.0	2.0	280.0	6.0	4.4	0.8
3*			100.0	2.0		
4*			140.0	5.0		
5			280.0	1.0		
6			280.0	6.6	20.0	0.08
7			220.0	4.4		
8			300.0	2.5		
<i>Without Caronamide</i>						
3			40.0	0.24		
5*			12.5	0.133		
7*			40.0	1.0		
9			20.0	0.133		

\*Injection made into tubing of continuous intravenous drip

It may be seen from Table I that substantial penicillin levels were achieved in the cerebrospinal fluid, especially when Caronamide was employed as an adjuvant. The levels in this group ranged from 1.0 to 6.6 units per milliliter of spinal fluid with an average of 4.0 units per milliliter. In two patients tested after the fifth injection a level of 2.0 units of penicillin per milliliter of spinal fluid was present. Penicillin was still found in the spinal fluid in the patients tested fourteen hours after the last injection.

In a number of instances the blood penicillin levels were not as elevated as had been anticipated. This occurred in two patients who received the antibiotic via the intravenous tubing through which a 10 per cent solution of glucose was being administered. It is believed that such factors as dilution and loss of penicillin because of diuresis may have played a role. In all the patients who received intermittent injections and Caronamide, the blood penicillin levels were markedly elevated as previously reported.<sup>1</sup> Considerably less penicillin was found in the blood and spinal fluid of those patients who did not receive Caronamide.

These studies demonstrate that substantial penicillin levels can be attained in the cerebrospinal fluid of patients with normal cerebrospinal systems. These levels are presumably due to the elevated blood levels and not to a change in permeability of the meninges. However, it is possible that even higher levels

\*Obtained from the United States Department of Agriculture



might be attained in the presence of diseased and more permeable membranes. In such cases intrathecal administration of penicillin in the treatment of the infections might prove unnecessary.

#### SUMMARY

High penicillin levels in the cerebrospinal fluid were produced in normal individuals by a method designed to produce high sustained blood levels. In three instances the presence of the antibiotic was still demonstrable in the spinal fluid fourteen hours after the last intravenous injection of penicillin.

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# THE EFFECT OF SODIUM SALICYLATE UPON SERUM DISEASE IN RABBITS

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## INTRODUCTION

THE therapeutic value of salicylates in the treatment of acute rheumatic fever and serum sickness has long been recognized,<sup>1 2</sup> but most clinical experience has indicated that these drugs have no specific effect upon the disease processes.<sup>1, 3 5</sup> There has been little opportunity however to observe whether the lesions of these diseases are altered by salicylate therapy. Although an experimental approach would be helpful in evaluating the histopathologic effects of these drugs, in the past this type of study has been impossible because of the absence of a suitable counterpart of rheumatic fever or serum sickness in animals. Recently such an opportunity has been offered by the production of serum disease in rabbits<sup>6</sup> and the demonstration that it is characterized by lesions of the arteries and heart which are similar in several respects to those of acute rheumatic fever.<sup>9</sup>

The following experiments were undertaken, therefore, to test the influence of sodium salicylate therapy upon the lesions resulting from the injection of rabbits with large intravenous doses of normal horse serum. Because a previous study had suggested that the necrotizing arteritis which some rabbits develop following horse serum injection might be due in part to arterial spasm,<sup>10</sup> an opportunity was taken to study the arterial blood pressures of some of the animals during the course of development of the lesions. In addition the erythrocyte sedimentation rates of the rabbits were measured, since preliminary observations indicated that they became elevated during the course of serum disease in the rabbit as in man. It was hoped that these determinations might give a clinical measure of the severity of the developing lesion where other criteria had failed. We also wished to ascertain the effects, if any, of salicylate therapy upon the increased sedimentation rates.

## EXPERIMENTAL PROCEDURE AND MATERIALS

Two experiments were performed. In the first, fifteen rabbits were used. During a preliminary period of eight days before the first injection of horse serum, four determinations of each animal's blood pressure and sedimentation rate were made to establish normal values. On the first day of the experiment all but two of the rabbits were injected with 10 ml (0.5 kilogram) of horse serum intravenously. The two uninjected animals served as controls for the blood pressure determinations and the other for salicylate therapy. Arterial blood pressures and erythrocyte sedimentation rates, the latter always accompanied by cell counts

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determinations, were measured on alternate days throughout the experiment. On the sixth day following the initial injection of horse serum a number of the rabbits began to show elevated sedimentation rates and erythematous ears<sup>6</sup> suggesting a developing serum reaction. Sodium salicylate treatment was then begun in half the injected rabbits and one of the un-injected controls. In the treatment of acute rheumatic fever Coburn advocated maintaining plasma salicylate levels above 350 gamma per milliliter. Because this average level in rabbits almost always results in severe toxicity, the drug was administered in quantities designed to maintain an average of about 250 gamma per milliliter throughout the twenty four hour period. The animals were paired so that those showing similar sedimentation rate increases were divided between the salicylate treated and the untreated groups. Salicylate therapy was continued through the tenth day when it was assumed that the toxic reaction to the first injection of horse serum was complete.<sup>6</sup> On the sixteenth day of the experiment salicylate therapy was resumed in the treated rabbit. Since in our experience arteritis has been produced in greater intensity and in more animals following a second large injection of horse serum all of the animals were reinjected intravenously on the seventeenth day of the experiment with 10 ml per kilogram. Salicylate therapy was continued for the following eleven days at the end of which time the treated rabbits and their controls were sacrificed and autopsies were taken for histologic study.

Twenty two rabbits were used in the second experiment which was similar in plan to the first with the following exceptions. Blood pressures were not determined. Sedimentation rates and hematocrits were each measured twice before the experiment started five, seven, nine, twelve, and fourteen days after the first injection of horse serum and two, four and six days after the second injection. Eighteen rabbits were injected with horse serum, three received salicylate only and one served as a control on the effects of bleeding. Ten rabbits of the group injected with horse serum were treated with salicylate beginning on the sixth day and treatment was continued in the survivors until the experiment ended. A second injection of horse serum was given on the sixteenth day of the experiment and the survivors were sacrificed on the twenty second day.

**Animals**—Thirty seven adult rabbits of both sexes were used. With the exception of four animals they were all albinos. Their initial weights varied from 1.9 to 3.9 kilograms. They were caged singly and fed a commercial rabbit food and water ad libitum.

**Horse Serum**—Two lots of normal horse serum without preservatives were used for each experiment. An aliquot of each was cultured immediately before it was injected and was found to be sterile. The serum was injected slowly into a lateral ear vein using sterile precautions.

**Sedimentation Rates**—These were determined using thick walled capillary tubes with the specifications of Westergren.<sup>1</sup> The tubes were filled to a 20 cm mark and their lower ends were plugged with modeling clay. They were kept in a vertical position by the method suggested by Foster.<sup>12</sup> Dry oxalate mixture<sup>14</sup> served as the anticoagulant. Hematocrits were always determined simultaneously by a micromethod.<sup>1</sup> The sedimentation rate readings were made at two hours and were corrected for anemia with data obtained in this laboratory by Fusibuehne<sup>15</sup> who employed this technique with the same strain of rabbits. With this method the healthy rabbit with a normal hematocrit has a two hour sedimentation rate of less than 5 millimeters.

**Arterial Blood Pressures**—These were measured using the method and apparatus described by Grunt and Rothschild.<sup>17</sup> The same point on the same ear artery was used for each determination. The ear was always warmed until there was maximal vasodilatation in front of the light which served as the source of illumination for the readings. Then three readings were made in rapid succession and the average was recorded as the blood pressure. Both systolic and diastolic readings were obtained.

**Sodium Salicylate Administration**—Preliminary experiments indicated that it was necessary to give a total quantity of 750 mg per kilogram in divided doses each day in order to maintain salicylate levels of approximately 250 gamma per milliliter or above throughout most

Rockland Rabbit Clets

kindly supplied by Dr. F. G. Jones of the Lilly Research Foundation Indianapolis Ind

of the twenty four hour period. In the first experiment the salicylate was administered to each treated rabbit in three divided doses of 250 mg per kilogram at 8:30 A.M., 1:30 P.M., and 10:30 P.M. Two routes of administration were tried in this experiment. On days seven, eight, and nine a 5 per cent solution of sodium salicylate in distilled water was fed by stomach tube. Because of technical difficulty in passing the tube and the death of one of the rabbits from tracheal passage, the drug was given subcutaneously in a 4 per cent solution on the tenth day. When therapy was resumed on the sixteenth day the subcutaneous route was again used and continued until the twenty eighth day. An occasional dose was omitted if the animal appeared toxic.

In the second experiment, a 5 per cent aqueous solution of sodium salicylate was given by stomach tube throughout the treatment period. This route was chosen because further observations had indicated that a more sustained level could be attained in this fashion and further experience had made this route safer. Although the times of administration were similar to those of the first experiment, the amount of drug was varied from rabbit to rabbit and from day to day as follows. A total of 850 mg per kilogram divided into three doses was given for the first twenty four hours. Thereafter the daily quantities were adjusted according to the plasma levels obtained in an attempt to maintain comparable level. The divided doses varied between 75, 75, and 100 mg per kilogram per day and 300, 300, and 300 mg per kilogram per day. The larger amount always was given at 10:30 P.M. in an attempt to maintain a high level over the ten hour period at night.

*Plasma Salicylate Determinations*.—These were made according to the colorimetric method of Brodie, Udenfriend, and Coburn<sup>18</sup>. Readings were taken with a Klett Sumner on photoelectric colorimeter using a filter with a wave length of 540 millimicrons. On the days when determinations were made on the treated rabbits, an equivalent amount of blood was drawn from the untreated rabbits in order to equalize blood loss.

*Antibody Titers*.—Serum was collected on the sixteenth day in the first experiment and on days seven, fourteen, and twenty one in the second experiment from all the rabbits. This was stored in paraffin sealed tubes at  $-20^{\circ}\text{C}$  until the end of the experiment when precipitation titers to horse serum were determined by the collodion particle agglutination technique of Cannon and Marshall<sup>19</sup>.

*Histologic Studies*.—The animals which survived were sacrificed by air embolism. In the first experiment a single transverse section through the base of the heart was made, in the second, four sections of the heart were made, one through each of the four valve rings. In addition, in each experiment sections of the lungs, mediastinum, diaphragm, liver, stomach, pancreas and spleen, mesenteric lymph nodes, left kidney, left adrenal, bone marrow, and testis or uterus and ovary from each rabbit were examined. All tissues were fixed in Zenker formalin solution, embedded in paraffin, and stained with hematoxylin and eosin.

## EXPERIMENTAL RESULTS

*Sedimentation Rates*.—Fig. 1 shows the corrected two hour erythrocyte sedimentation rates and the mean rates found in the treated and untreated groups in the second experiment. The changes in the sedimentation rates in the two experiments were so similar that only those of experiment two are given in detail. Normal rates persisted in the salicylate treated and bleeding control animals which did not receive intravenous horse serum. Of the thirty one animals given horse serum only five failed to show a rise above 20 mm after the first or second injection. The rate usually began to rise from the fifth to the seventh day at the time when the first manifestations of hypersensitivity were seen. A further elevation usually occurred one to two days after the second injection of horse serum when presumably the extent and severity of the lesions were increasing. Salicylate treatment did not exert any notable effect on the sedimentation rate in spite of the fact that the lesions were less severe in this group of animals.

No lesions except those attributable to hypersensitivity could be found at autopsy to account for the observed change of the sedimentation rate.

It should be noted that all of the rabbits showed a decreased cell volume. The average hematocrit of twelve salicylate treated animals fell from 42.0 to 25.8 per cent, while the average of twelve untreated rabbits fell from 41.0 to 30.7 per cent. Similarly, the hematocrits of the rabbits receiving salicylate alone dropped about twice as much as those of the bleeding controls. It appears that salicylate in this dosage increases the anemia which rabbits receiving large doses of horse serum develop.

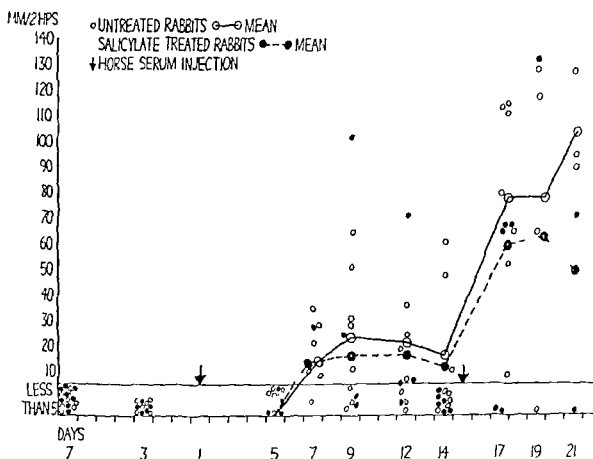


Fig. 1—Erythrocyte sedimentation rates of rabbits injected with horse serum

**Blood Pressure Findings**—For purposes of comparison the nine rabbits which received two injections of horse serum and survived to the end of the first experiment were divided into three groups (Fig. 2). Group A consisted of three rabbits which at death manifested severe arteritis. Group B was composed of three similarly injected rabbits which developed minimal or no arteritis and Group C the three rabbits which received both serum and salicylate and which also showed only slight arterial reaction. Only the systolic pressures are recorded in the graph since the changes in the diastolic pressures were parallel.

Sustained hypertension was not noted in any animal of these three groups. It is of interest however that there was a definite trend for the arterial pressures of the untreated groups (A and B) to rise following the first injection of horse serum. However this was no more marked in the rabbits which later exhibited lesions than in those which reacted with little or no arteritis. This elevation was most evident from day seven to day eleven following injection an interval cor

responding to the manifestations of serum disease in the rabbit, namely ear edema,<sup>6</sup> temperature rise,<sup>20</sup> and rise in sedimentation rate (Fig 1). On the other hand the treated rabbits which were receiving salicylate during this period (day seven through day ten) showed a sharp drop in blood pressure. When salicylate therapy was stopped there was a rise toward the preinjection level.

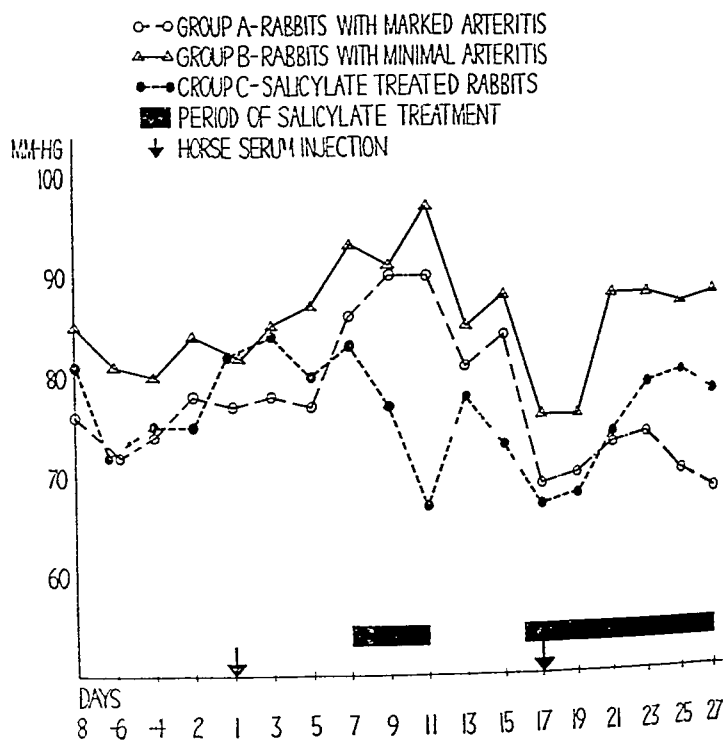


Fig. 2—Mean systolic blood pressures of rabbits injected with horse serum.

The second injection of horse serum on the seventeenth day was accompanied by a fall in the arterial blood pressure which was most apparent in the two untreated groups, A and B. The remainder of the observation period was characterized by a gradual rise of the arterial pressure in Groups B and C to values comparable to the preinjection levels (Group C) or slightly higher (Group B). The rabbits which were developing marked generalized arteritis (Group A) showed no significant change in their blood pressure from day nineteen to day twenty-seven. Furthermore the drop in blood pressure observed in the salicylate treated group in the first treatment period did not recur during this course of therapy.

**Salicylate Levels**—Maintenance of adequate salicylate levels was difficult because of wide variation in the animals' tolerance for the drug. In spite of careful adjustment of individual doses, seven rabbits died of salicylate intoxication before the completion of the experiments. The level was considered to be adequate during most of the twenty-four hour period if a plasma concentration of 150 gamma per milliliter or more was present five hours after the previous administration of the drug. In the first experiment plasma salicylate levels were

determined on days ten and eighteen at six hours and on the twentieth day at three hours after the morning dose. These levels were always above 150 gamma and the average of the three determinations in each animal ranged between 343 and 544 gamma. In the second experiment plasma salicylate concentrations were measured on the eighth day twelve hours after the evening dose of drug and on days nine, twelve, fourteen, sixteen and nineteen five hours after the morning dose. Individual values ranged between 0 and 530 gamma. In four animals, average five hour levels were between 254 and 353 gamma. The other four animals had average five hour readings of 90 to 128 gamma but were receiving such large amounts of salicylate (0.9 to 1.1 gm per kilogram per day) that it did not seem wise to increase the dose further.

*Histopathologic Findings*—Table I summarizes the findings in experiment one in four treated and four untreated rabbits which were paired on the basis of similar responses in sedimentation rate following the first injection of horse

TABLE I EXTENT AND SEVERITY OF THE LESIONS OF SERUM DISEASE IN TREATED AND UNTREATED PAIRS OF RABBITS (EXPERIMENT ONE)

HORSE SERUM AND SODIUM SALICYLATE					HORSE SERUM				
RABBIT	DAY OF DEATH	HISTOLOGIC LESIONS			RABBIT	DAY OF DEATH	HISTOLOGIC LESIONS		
		MYO CARDIAL	ARTE RIAL	RENAL			MYO CARDIAL	ARTE RIAL	RENAL
1	17	0	0	0	4	17	0	+++	0
2	28	0	+	0	1	28	0	++++	±
6	28	0	++	±	1	28	0	++++	0
8	28	0	0	0	14	28	0	±	0

serum. One pair died of an anaphylactic shock immediately following the second injection of horse serum and the other three pairs survived to the end of the experiment. The lesions were graded separately by two observers from 1 plus to 4 plus depending upon their extent and severity. The extent of the valvular lesions could not be evaluated in this experiment because of the manner in which the heart sections were made. It should be noted that the numbers of animals to be compared were small in this experiment because two salicylate treated rabbits died on days eight and nine. Despite the small series the results strongly suggest that salicylate treatment had depressed the developing arteritis in the salicylate treated group.

TABLE II EXTENT AND SEVERITY OF THE LESIONS OF SERUM DISEASE AND PRECIPITIN TITERS TO HORSE SERUM AT DEATH IN TREATED AND UNTREATED PAIRS OF RABBITS (EXPERIMENT TWO)

HORSE SERUM AND SODIUM SALICYLATE							HORSE SERUM						
RAB BIT	DAY OF DEATH	HISTOLOGIC LESIONS					RAB BIT	DAY OF DEATH	HISTOLOGIC LESIONS				
		VAL VULAR	MYO CAR DIAL	ARTE RIAL	RENAL	ANTI BODY TITER			VAL VULAR	MYO CAR DIAL	ARTE RIAL	RENAL	ANTI BODY TITER
49	15	0	0	0	0	—	58	15	+++	0	++	+	—
59	18	0	0	0	0	—	55	18	++	0	0	0	1 160
60	18	0	+++	0	0	1 160	56	18	++++	++++	+	±	1 640
53	19	±	0	0	0	1 80	54	19	±	+++	0	0	1 160
41	19	++	0	+	0	1 160	48	19	+++	+++	++	+	1 1280
41	—	±	0	0	0	1 160	49	22	++	0	+	+	1 2560
42	22	±	0	±	0	1 640	46	22	++	0	++	0	1 2560
—	22	±	0	±	0	1 640	43	22	±	0	+	0	1 320

40



9



60



53



Fig 3—Mitral valves of rabbits treated with sodium salicylate (Rabbits 45 59 60 and 53) compared with their untreated controls (Rabbits 58 55 56 and 54) Magnification  $\times 100$



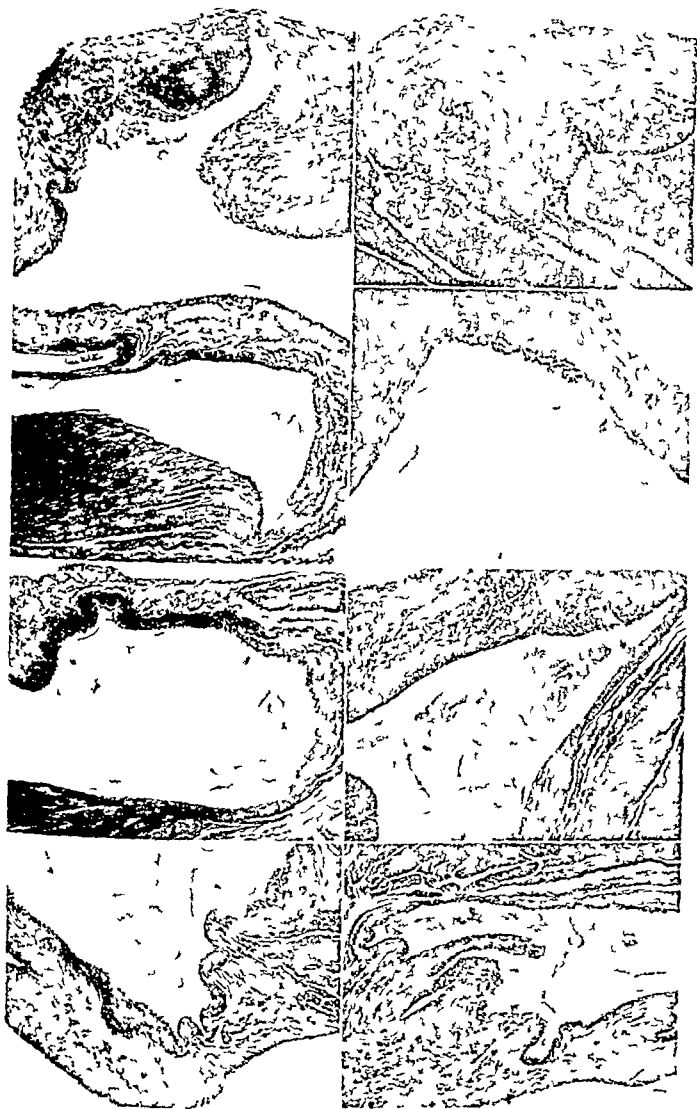


Fig 4—Mitral valves of rabbit treated with sodium allylate (Rabbits 4, 41, 42, and 44) compared with their untreated controls (Rabbits 18, 19, 40, and 43). Magnification 100x.

Table II summarizes for experiment two the histologic findings in equal treated rabbits paired with eight untreated rabbits on the basis of the rise in the sedimentation rate after the first injection of horse serum. Two salicylate treated rabbits which had been injected with horse serum died early, one of false passage of the stomach tube and one of salicylate intoxication. These were eliminated from the experiment. Five salicylate treated animals died later, one of false passage of the stomach tube and four of salicylate intoxication. One died on day fourteen, two on day eighteen, and two on day nineteen. In each instance the paired untreated control animal was sacrificed on the same day.

It may be seen from Tables I and II that the lesions of the salicylate treated rabbits were considerably less severe and extensive than those observed in the untreated group. This fact is further emphasized by Figs 3 and 4 which illustrate the changes in the mitral valves in each pair of rabbits in experiment two. For reasons which are not clear there was a much greater degree of necrotizing arteritis in the animals of experiment one, while the lesions observed in the second experiment were more severe in the myocardium and heart valves.

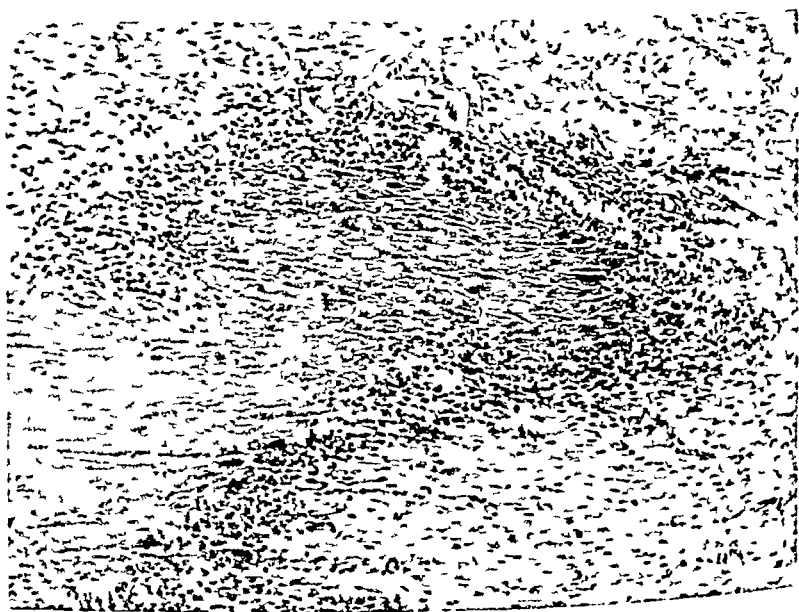


Fig. 5—Area of necrosis in the right ventricle of Rabbit 60. Magnification  $\times 140$ .

Microscopically the arteritis, when present, usually was seen in the medium sized arteries of the myocardium, lungs, pancreas, mesentery, stomach, kidney, liver, adrenal, diaphragm, and testis or uterus. Valvular changes, mostly proliferative in type, were found predominantly at the base of the valve leaflets. The latter were confined almost entirely to the mitral and aortic valves as they are in rheumatic fever. These lesions were all similar qualitatively to those described by Rich and Gregory.<sup>7-9</sup>

An additional lesion was noted in the myocardium, to our knowledge, that has not been reported previously in rabbits with serum disease. The lesion con-

sisted of many areas of necrosis of varying size occurring almost exclusively in the myocardium of the right ventricle (Fig 5). In no instance did we observe thrombosed arteries in the hearts which showed these lesions. Although the myocardial fibers in the areas were disrupted and undergoing necrosis there was a notable absence of infiltration by inflammatory cells. In the older lesions there was early organization so that the whole area was composed of a network of large mononuclear cells. These appeared to be somewhat abnormal large macrophages or young fibroblasts with clear chromatin poor nuclei and large deeply staining nucleoli (Fig 6). The lesions were not seen in the animals which did not receive horse serum.

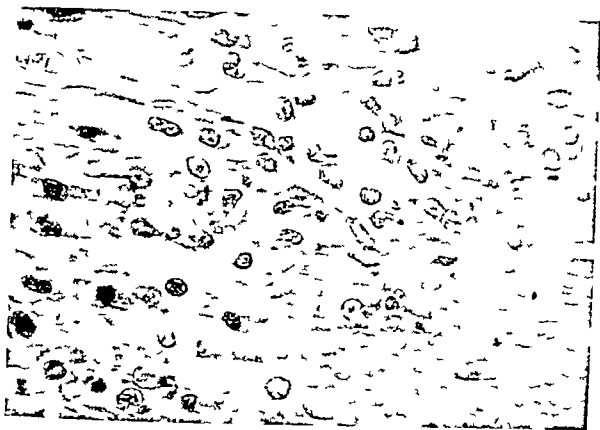


Fig 6—Higher magnification of an area at the edge of the lesion in Fig 5. Magnification  $\times 500$ .

The renal lesions usually were localized to the point where afferent arterioles entered glomeruli. At that point there was an apparent proliferation of endothelial cells or cells of the juxtaglomerular apparatus accompanied by a variable infiltration of lymphocytes. No significant changes were seen in the glomerular tufts. These lesions were mild and were observed in only a few animals (Tables I and II).

Except for pulmonary hemorrhage and edema and glycogen depletion of some of the livers there were no changes in the tissues of the salicylate treated animals or the salicylate controls which could be attributed to toxic effects of the drug.

**Antibody Titers**—Titers of sera obtained from the rabbits sixteen days after horse serum injection in experiment one and seven and fourteen days after injection in experiment two were similar in the treated and untreated groups. Appreciable differences in precipitin titers between the salicylate treated and untreated rabbits occurred in sera drawn eighteen to twenty two

days after the first injection of horse serum in the second experiment (two to six days after the second injection). These are recorded in Table II where it can be seen that with one exception higher titers were recorded for the untreated member of each pair. The only pair of rabbits in which this difference was not observed (Rabbits 57 and 43) showed the least contrast in the severity and extent of their lesions.

#### DISCUSSION

The blood pressure observations do not support the concept that the arterial lesions develop as a result of arterial spasm<sup>10</sup> or that hypertension is the important common factor in the pathogenesis of experimental periaarteritis.<sup>21</sup> This is in agreement with the experimental results of Hopps and McCollum<sup>22</sup> who failed to find a correlation between blood pressure changes and the lesions occurring in rabbits receiving large doses of horse serum. The transient elevation of blood pressure following the initial injection of horse serum might be interpreted as a manifestation of a generalized arterial constriction. Its inconstant occurrence following the second injection of serum and the lack of correlation of the rise with the development of arterial lesions make it untenable to assign to hypertension any significant role in the developing arteritis. On the other hand, the observations do not disprove the hypothesis that local arterial spasm may occur and be of some importance in the development of the arterial lesions. It is possible that the medial ear arteries utilized for the blood pressure determinations were unsuitable to reflect a generalized vasospastic reaction. For the present the question of this phase of the pathogenesis of these arterial lesions must be left unanswered.

The erythrocyte sedimentation rate appears to offer a clinical measure of the developing lesions of hypersensitivity although the extent and severity of the lesions found do not always correlate with the degree of rise in sedimentation rate. Contrary to the clinical findings of some observers,<sup>23, 24</sup> sodium salicylate exerted little if any suppressive effect on the erythrocyte sedimentation response.

In these experiments an attempt was made to give the rabbits massive doses of salicylate comparable to those advocated by Coburn for human beings.<sup>11</sup> However, as had been noted previously in patients given large doses of salicylates, the quantities necessary to maintain a high salicylate blood level vary widely. It is extremely difficult to maintain a sufficiently high level in the rabbit without producing severe toxic reactions which often terminate in death. It appears from the results that the salicylate blood levels were adequate in most instances, although levels of 150 gamma or less were noted at some period of the twenty-four hours in many of the animals.

It is obvious that the severe toxic reactions which some of the rabbits exhibited as well as the anemia which most of them developed may have acted in a nonspecific fashion to prevent the development of the lesions. It should be noted however that the contrast between the lesions of the treated and untreated rabbits was not limited to those which showed toxic symptoms. Furthermore when an animal died with salicylate intoxication it usually had been noticeably sick for only a few hours, whereas the lesions appeared considerably older. In

will be important nevertheless to ascertain whether the lesions of rabbit serum disease can be prevented or treated with doses of salicylate which do not produce toxicity.

At present one can only speculate as to how salicylates produce their effects. It has been reported that salicylates depress antibody formation.<sup>28, 29</sup> Other writers do not agree.<sup>30, 31</sup> Our studies suggest that antibody formation was somewhat diminished in the salicylate treated group late in the experiment. However, since the concentration of antibody seems to have little correlation with development of the lesions,<sup>1</sup> the significance of this finding is questionable. Recent work by Dorfman and co-workers<sup>32</sup> confirms that of Ueffer<sup>31</sup> and suggests that sodium salicylate inhibits the spreading effect of hyaluronidase.

The question of whether salicylates merely suppress the symptoms or whether they exert a specific influence upon the lesions of rheumatic fever has been reopened recently by Coburn.<sup>33</sup> He has emphasized the possible specific action of large doses of salicylates in suppressing the rheumatic process and preventing the ensuing carditis. A few confirmatory studies have emphasized the need to administer the massive quantities of salicylate early in the course of the disease.<sup>32, 34</sup> However, Keith and Ross<sup>4</sup> found that even early treatment with large doses of salicylate did not hasten restoration of the sedimentation rate or diminish the incidence of subsequent heart damage as compared with early treatment with small doses of the drug. Others have reported that the intensive salicylate therapy recommended by Coburn did not alter the inflammatory reaction of rheumatic fever in the joints<sup>3</sup> or heart<sup>5</sup> in patients of whom most had had rheumatic fever for four or more weeks before treatment. Similarly, Wegria and Smull<sup>35</sup> found the duration of rheumatic attacks to be unaffected by massive doses of salicylate but suggested that early treatment might have proved efficacious. At present, then, the clinical value of maintaining high salicylate levels in the treatment of acute rheumatic fever is still on trial. It appears however that if salicylates are to produce a specific effect upon the lesions of rheumatic fever they must be given early in the course of the disease.

In contrast to the findings of Thomas and Stimmgeld<sup>3</sup> our results show that sodium salicylate, given in large doses early in the course of serum disease in rabbits, reduces the extent and severity of the resulting lesions. It must be emphasized that in these experiments salicylate therapy was begun six days after the initial injection of horse serum at a time when the lesions of hypersensitivity are presumably just beginning to develop.<sup>36</sup> It was continued throughout the period following the second injection of horse serum when it is assumed that the most marked tissue reactions occur. Because of its insidious onset the first attack of rheumatic fever usually is seen by the clinician at a comparably early stage. However, Schlesinger<sup>37</sup> and Coburn and Moore<sup>38</sup> have reported success in suppressing the rheumatic process by prophylactic administration of salicylate following streptococcal infection in subjects with previous rheumatic episodes. Further experiments are indicated to determine how long, following the injection of rabbits with horse serum the salicylate therapy can be initiated and still exert its suppressive effect upon the development of the lesions.

Many have called attention to the clinical and pathologic similarities between acute rheumatic fever and serum sickness,<sup>39</sup> and in our opinion rabbit serum disease represents the closest approximation to acute rheumatic fever yet produced experimentally. Despite these attractive similarities, our experience indicates that rabbit serum disease does not fulfill all of the rigid pathologic criteria listed by Gross and co-workers<sup>40</sup> for the experimental production of rheumatic fever. The Aschoff-like lesions are not numerous. Although their cellular components and location are typical they seldom show fibrinoid necrosis. Pericarditis is very rare and when present it is usually focal and minimal. Verrucae are uncommon in the valvular lesions and we have been unable to produce chronic valvular lesions like those in rheumatic fever by many repeated injections of horse serum over long periods of time.<sup>41</sup> Therefore at present one must be cautious in assuming that the inhibitory effect of salicylates upon the lesions of rabbit serum disease can be applied to the lesions of rheumatic fever.

#### SUMMARY AND CONCLUSIONS

The histopathologic lesions produced in twelve rabbits receiving two intravenous injections of sterile normal horse serum fifteen to sixteen days apart in doses of 10 ml per kilogram have been compared with those produced in twelve similarly treated rabbits which also received large doses of sodium salicylate starting six days after the first injection of horse serum. Blood pressures were determined repeatedly in some of the rabbits (experiment one). Sedimentation rates and blood salicylate levels also were followed.

It is concluded that

The arterial blood pressure of rabbits receiving large doses of horse serum showed no sustained elevation, and no evidence was obtained that a generalized arterial constriction plays a part in the pathogenesis of the arteritis of rabbit serum sickness.

The erythrocyte sedimentation rates of rabbits given large intravenous injections of horse serum appear to offer a clinical measure of the developing lesions of hypersensitivity. Although the degree of rise could be correlated to some extent with the severity of the lesions found, this correlation was not constant.

It is difficult to maintain salicylate levels of 350 gamma per milliliter recommended for man without producing intoxication and death in rabbits.

A moderate depression in the concentration of circulating antibody to horse serum was noted in the salicylate treated rabbits of the second experiment as compared with the untreated controls. This occurred eighteen to twenty-two days after the first injection of horse serum. Antibody concentrations earlier in the experiments were not significantly different in the treated and untreated groups.

A lesion of the right myocardium which occurred more frequently in the untreated rabbits is described. This consisted of focal necrosis accompanied by little inflammation.

The lesions seen in the salicylate treated rabbits, especially the arterial and valvular lesions, were greatly reduced in severity and extent as compared with those in the untreated animals

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# ALTERATIONS OF RADIAL OR BRACHIAL INTRA ARTERIAL BLOOD PRESSURE AND OF THE ELECTROCARDIOGRAM INDUCED BY TILTING

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INTEREST in the response of blood pressure and pulse rate to postural change was stimulated by the problem of rapid evaluation of tachycardia in military induction examinations. Preliminary observations indicated that a significant slowing of heart rate occurred with tilting to a head down position in subjects with tachycardia secondary to excitement. On the other hand this slowing did not occur in inductees with tachycardia which was later found to be due to organic disease. An increase of blood pressure occurred in all subjects during the tilt to the head down position. In this study careful analysis has been made of the response of heart rate and blood pressure to tilting in normal subjects in order to establish a standard to evaluate changed reactions due to disease or drugs.

Hill<sup>1</sup> first demonstrated the importance of gravity in the circulatory system of man. He also reported that the brachial systolic blood pressure as measured by the sphygmomanometer remained approximately the same on tilting from an erect to a head down position providing the readings were taken with the arm in the frontal plane perpendicular to the long axis of the body (horizontal plane). With the arm in this position the effect of gravity on the column of blood within the arteries of the arm remained constant.<sup>2</sup> These readings were not taken during or immediately following postural change but after the subject was in the altered position for an appreciable period of time. Wald, Cueney and Scott<sup>3</sup> using a Tycho's self recording sphygmomanometer found that following a positional change from horizontal to standing the brachial blood pressure fell below the level obtained in the recumbent position in about ten seconds and then regained or exceeded this level within thirty seconds. Their results with tilting from standing to horizontal position varied in the two subjects studied. Other attempts to analyze the brachial arterial blood pressure changes with tilting have resulted in widely divergent findings.<sup>4</sup> There are two important explanations for the discrepancies in these reports. (1) It is impossible to obtain accurate readings of a rapidly changing blood pressure with a cuff method and (2) the position in which the arm was maintained during the tilting has varied in the different experiments.

Slowing of the heart rate following a change from an erect to a horizontal or head down position has been described by numerous observers.<sup>5, 10, 11</sup> However these findings were interpreted from periodic pulse rate readings and the slowing was not consistently found in all normal subjects.

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In these studies we have recorded intra arterial blood pressure continuously with the arm maintained in a horizontal plane. Heart rate and rhythm have been interpreted from an electrocardiogram taken throughout the experiment.

### METHOD

Direct measurements of radial or brachial artery pressure during and after tilt have been obtained in more than two hundred subjects. This report details the findings of fifteen healthy young adults considered to have normal cardiovascular systems. Simultaneous electrocardiograms were obtained in these subjects, and in addition continuous electrocardiograms have been recorded in one hundred normal adults during and following tilting.

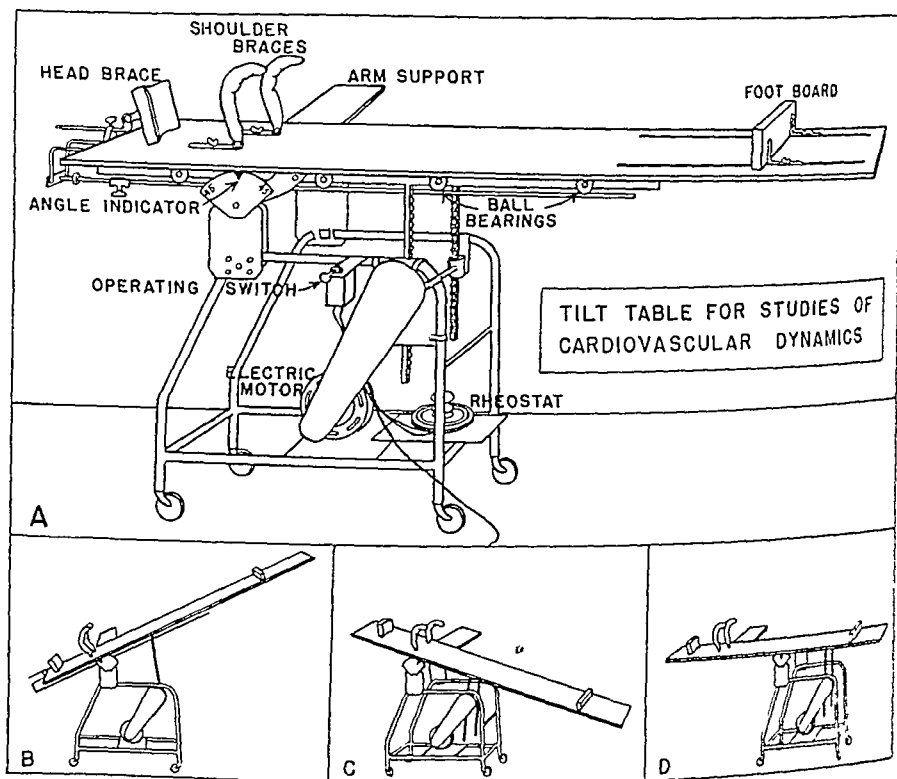


Fig 1—Tilt table for study of cardiovascular dynamics. A, horizontal position. B, 45 degree head-down position. C, 20 degree erect position. D, 15 degree erect position. Fulcrum at iliac crest region.

The subjects were tilted at a moderate rate from the 20 degree head up position to the 15 degree head down position. This position was maintained for at least fifteen seconds. The subjects then were returned to the original position.

Tilting was controlled by means of an electrically operated table (Fig 1). The subjects were securely supported so that muscular activity was not required to maintain the position.

The blood pressure tracings were determined by means of a Statham strain gauge adapted in our laboratory to record blood pressure changes by means of a string galvanometer electrocardiograph.<sup>12</sup> The gauge was ideally suited to this type of experiment. It was

\*Research Model Cambridge Electrocardiograph Cambridge Instrument Co. Inc. New York, N. Y.

compact (3 by 1 by 1 inch) and could easily be held in place during movements. It did not respond to external vibration. Changes of pressure caused an alteration of electrical output that was transmitted to the recording string galvanometer. This output was directly proportional to the pressure; thus linear records were produced.

Preliminary investigation indicated that this system had adequate frequency for accurate pressure recording.

In these experiments a size 20 1½-inch needle with a 45 degree sharp bevel was attached directly to the gauge. The system was completely filled with a solution of heparin (5 mg per cubic centimeter) and the needle with bevel directed toward the heart was inserted into the brachial artery at the elbow or into the radial artery at the wrist.

At least three sets of observations were made on each subject.

## RESULTS

### *Blood Pressure —*

*Head Down Tilt (20 Degrees Erect to 15 Degrees Head Down)* An elevation of arterial blood pressure in the arm occurred during the head down tilt in all of the subjects studied. In general the degree of rise was uniform throughout the tilt; the maximum blood pressure occurred immediately after cessation of motion.

In the subjects with normal cardiovascular systems this elevation of blood pressure (average rise 19 mm Hg systolic/16 mm Hg diastolic) was followed by a gradual fall lasting from eight to eighteen seconds until the blood pressure reached a level that was usually slightly higher than that obtained in the erect position. The average fall in the normal subjects during this period was 13 mm Hg systolic/14 mm Hg diastolic (Figs. 2 and 3).

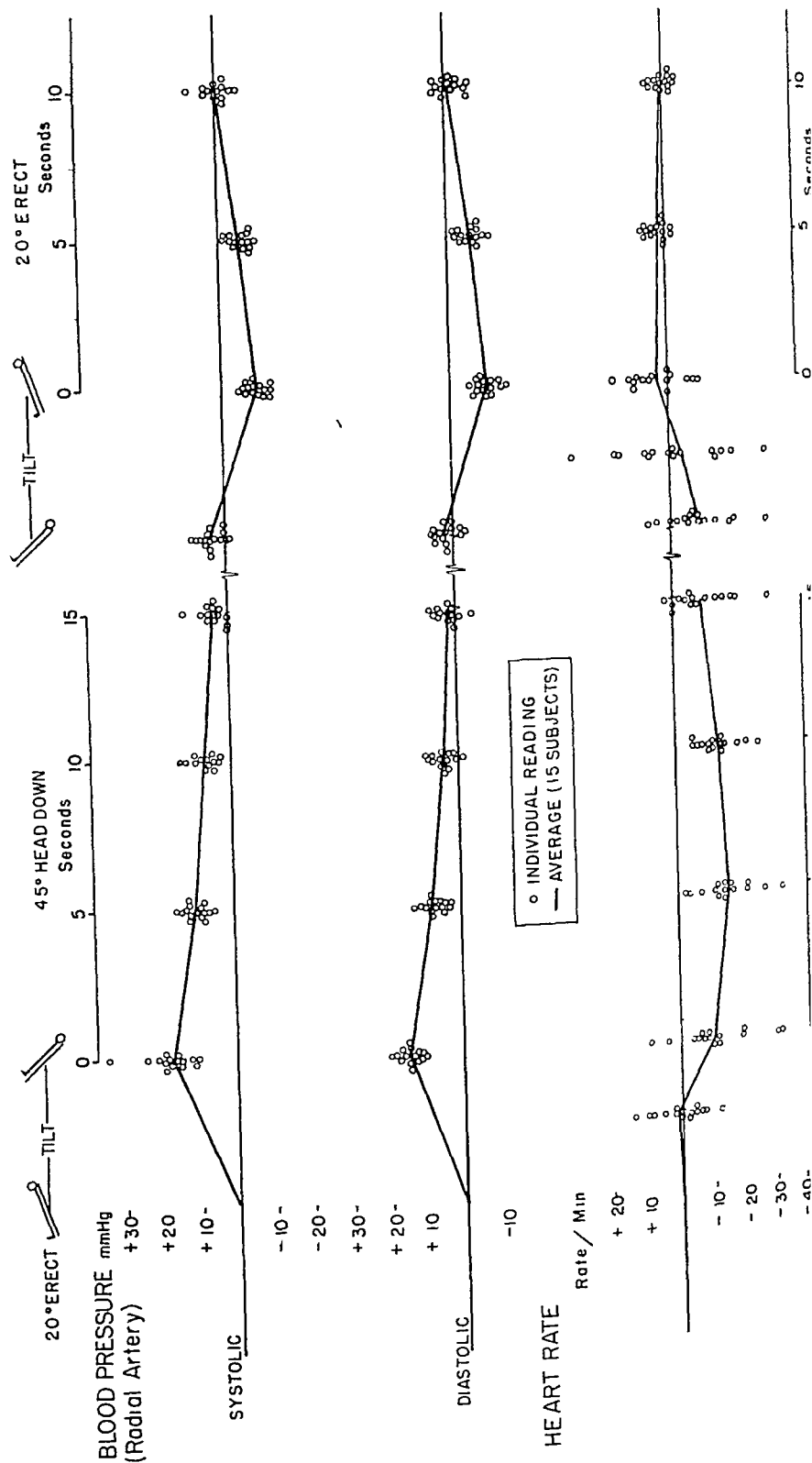
*Head Up Tilt (15 Degrees Head Down to 20 Degrees Erect)* During the return tilt the blood pressure fell in all of the subjects. This decline was usually uniform throughout the tilt; the lowest blood pressure occurred immediately after the cessation of motion.

In the normal subjects this primary fall of blood pressure (average 14 mm Hg systolic/13 mm Hg diastolic) reached a level below that initially obtained in the erect position, and it was followed by a rise; the blood pressure returned to the starting level within eight to eighteen seconds of the completion of the tilt (Figs. 2 and 3).

### *Heart Rate —*

*Head-Down Tilt* In subjects with normal cardiovascular systems slowing of the heart rate invariably occurred subsequent to the elevation of blood pressure. The rate for the five second period immediately following the onset of slowing was always less than the erect rate and less than 90 per minute (Figs. 2, 3, and 4). In the majority of cases the onset of slowing was abrupt, resulting in bradycardia which was maintained for a few beats; the rate then gradually increased. In others the slowing developed more gradually. Regardless of the type of the initial response the rate became relatively stable within about fifteen seconds of the completion of the tilt at a rate that was usually slower than previously recorded in the erect position.

*Head Up Tilt* The heart rate suddenly increased following the drop in blood pressure. This resulted in a rate that was more rapid than the initial



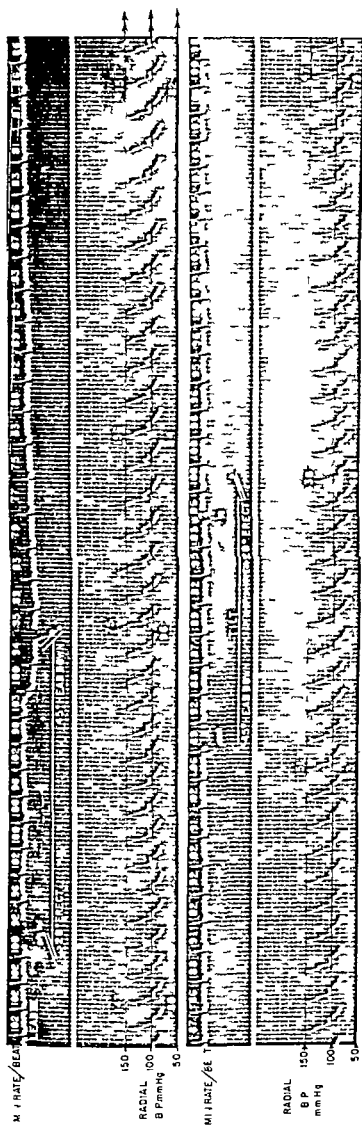


Fig. 3.—Initial intra-arterial pressure and heart rate recording (normal subject, F. J., 53-year-old colored man) Normal cardiovascular system. Con-  
tinuous radial intra-arterial pressure and electrocardiogram recorded during and after tilting to the head down position. The vertical white line marks the  
tenth second interval. Blood pressure during tilting to the head down position. The pressure fell from 143/80 to 115/96. With the position maintained  
for ten seconds it returned to 143/80. During the return of the blood pressure to 143/80 the heart rate fell from 151/8 to 136/88. With the  
return of the blood pressure to 143/80 the heart rate abruptly lowered to 84 per minute. In the five minutes following the onset of slow motion the  
rate then gradually increased stabilizing within fifteen seconds of the initial erect position. With the return of the drop of blood pressure  
the initial return to the range of the initial erect position.

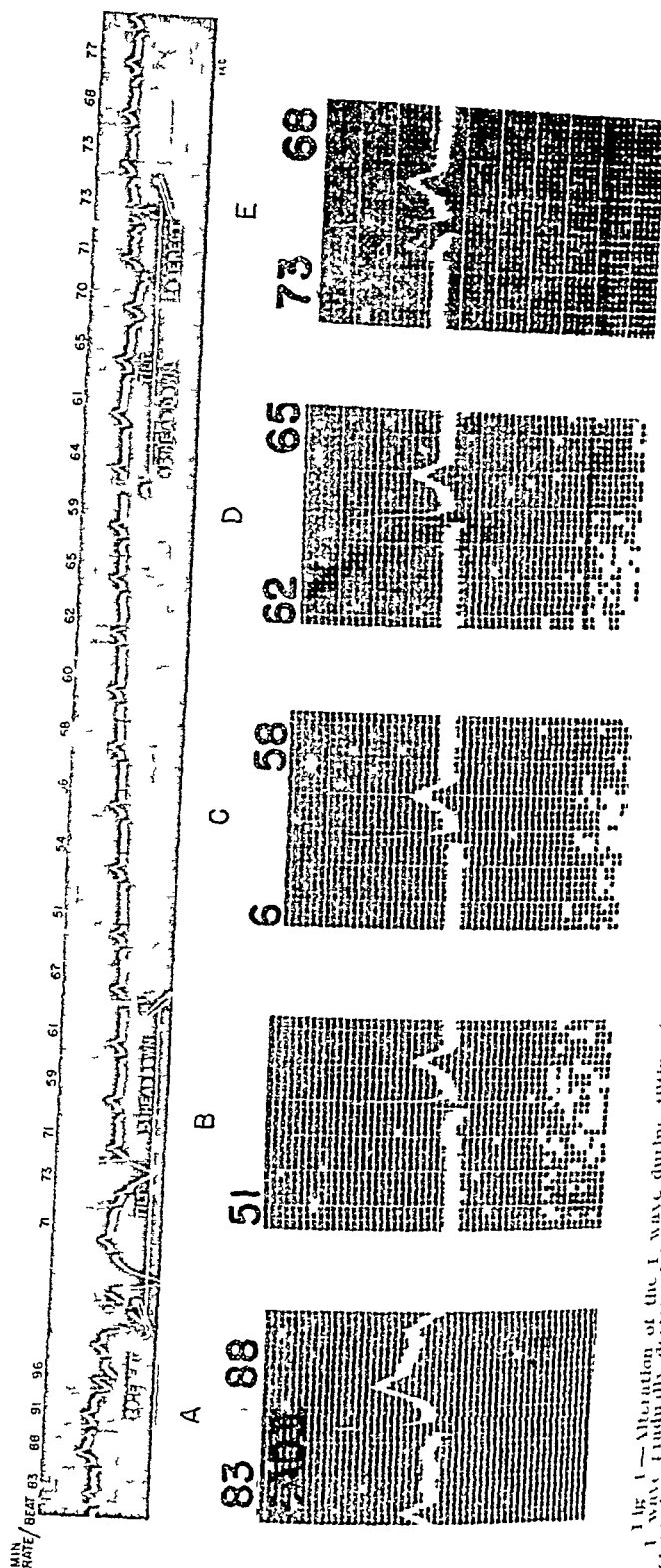


Fig. 1 — Alteration of the I-wave during fitting. (normal subject). In the erect position (A) P waves were present. In the head down position the I-wave gradually disappeared (B) and finally disappeared (C). The (R interval preceding the disappearance of the I-wave was the same as in the erect position. With the return of the I-wave to its initial appearance (D) the P-wave was again present. In the head down position the I-wave gradually disappeared (E).

rate in this position in about half of the subjects. In all instances the rate about ten seconds after the end of the tilt was essentially the same as the original rate (Figs 2, 3, and 4).

*Electrocardiogram*—Our findings on the effect of positional change on the PR interval, the QRS complex and the T wave of the normal electrocardiogram are in agreement with those reported by other investigators.<sup>13, 19</sup> However there are two observations not yet emphasized which we consider worthy of description.

(1) A slowing of the heart rate determined from prolongation of the R-R intervals followed the assumption of the 45 degree head down position in every normal subject.

(2) In twenty per cent of the normal subjects tilted to the head down position the P wave gradually decreased in size and finally disappeared for several beats (Fig. 4). The disappearance of the P wave was not preceded by a shortening of the P-R interval. This change did not invariably occur in the same individual on successive tilting.

#### DISCUSSION

*Blood Pressure*—The changes of the radial and brachial intra-arterial blood pressure recorded in these experiments were presumably a reflection of the blood pressure changes in the large arteries that arise from the aortic arch because the arm was maintained throughout the tilt in a position in which the force of gravity of the column of blood within the arteries of the arm remained constant. Our studies have shown that the blood pressure recorded from these arteries invariably rose during the tilt from a head up to a head down position and fell during the reverse tilt. In other experiments we have demonstrated that the blood pressure recorded from the femoral artery always fell during the tilt from the head up to the head down position and rose during the return tilt.<sup>2</sup> These findings indicated that the primary alterations of pressure that accompanied the postural changes were due to a shift of the hemostatic force of gravity of the column of blood enclosed by the aorta and its branches and were not secondary to reflex changes. These primary alterations of blood pressure apparently acted as intra-arterial stimuli to the regulatory mechanism that controls blood pressure.

In the subjects with normal cardiovascular systems these primary alterations of radial or brachial pressure were followed by a characteristic pattern of depressor response in the head down position and of pressor response in the erect position whereby the blood pressure in the region of the aortic arch returned within eight to eighteen seconds to a level that was approximately the same regardless of the position of the body.

The mechanism of blood pressure regulation has been explored extensively in animals. An elevation of arterial blood pressure stimulates the proprioceptors of the carotid sinus region,<sup>1</sup> the left ventricle, the aortic arch, the thoracic aorta,<sup>3, 24</sup> and the abdominal aorta. This induces reflex dilatation of the splanchnic and peripheral arterioles<sup>1, 3</sup> and of the large thoracic vascular

bed<sup>2c</sup> intracranial-extracranial anastomoses,<sup>27</sup> and slowing of the heart rate,<sup>1</sup> resulting in a return of the blood pressure to its previous level. A fall in arterial pressure induces a diametrically opposite type of vasomotor response.

The presence of both pressor and depressor regulatory responses in human subjects with normal cardiovascular systems has been demonstrated in this study. However, exact analysis of these responses in man awaits further investigation of the sites of initiation, the mechanisms of regulation, and the physical factors that determine the degree of blood pressure rise that occurs during tilting. That man has vascular reflexes which influence blood pressure has been proved through the results of denervation operations and by the fact that stimulation of the carotid sinus area may produce both bradycardia and fall in blood pressure. Evidence that reflexes are important in the postural control of blood pressure is found in the peripheral dilatation<sup>30-31</sup> and the bradycardia that occur on assumption of a head-down position.

The function of the regulatory mechanisms that maintain the pressure in the upper aorta at a relatively stable level can be most clearly thought of as a reaction to protect the brain (and probably the heart) against arterial pressure changes that might harm the individual. At one extreme a marked elevation in intracranial arterial pressure might conceivably result in brain damage and at the other a lowering of cerebral arterial pressure might lead to cerebral anoxia and syncope.

*Heart Rate*.—In a normal subject an inverse relationship of heart rate to blood pressure has always followed the change of blood pressure produced by tilting. Similar alterations in heart rate have been noted subsequent to the change of blood pressure that occurs during and after the Valsalva experiment and following the occlusion or opening of arteriovenous fistulas.<sup>33</sup>

The relation between the position of the body, the blood pressure, and the heart rate in these experiments suggests that the change of rate is one of the reflex mechanisms that regulates blood pressure. In the head down position the initial slowing greatly resembled a braking action. In addition the stabilized rate in this position was characteristically slower than the rate in the erect position even though the blood pressure had returned to the erect level. This indicated that this relative bradycardia was one of the means whereby the potential increase of pressure due to the weight of the column of blood (now directed toward the upper arterial regions) was counteracted.

Although it has been known for many years that slowing of the heart may occur in the head down position, the universal appearance of this reaction in subjects with normal cardiovascular systems has not been appreciated. This is probably due to the fact that the slowing is often of short duration and is detected only through a continuous method of recording.

The disappearance of the P wave for a few beats in a large proportion of normal subjects tilted to the head-down position cannot be explained at present. The fact that the disappearance was preceded by a gradual decrease in amplitude of the P wave and was followed by a gradual increase in amplitude



evidence that position alone was not the responsible factor. The fact that the changes of the P wave were not associated with a shortening of the P R interval indicates that the resultant rhythm was not nodal.

In 1897 Hill<sup>3</sup> concluded from his experimental work on the blood pressure and heart rate of animals that the effects of changing the position afford a most delicate test of the vasomotor mechanism. The development of a suitable method of blood pressure recording has made it possible for us to demonstrate that this conclusion is probably valid for human subjects. We feel that this method of study may prove to be of value in obtaining a better understanding of the normal physiology of cardiovascular reflexes and of the influence of disease and drugs on these responses.

#### SUMMARY AND CONCLUSION

A graphic record of radial or brachial intra-arterial blood pressure during and after tilting at a moderate rate between the 20 degree erect and the 45 degree head down position was obtained in more than two hundred subjects. In this study careful analysis was made of the response of heart rate and blood pressure to tilting in fifteen healthy young adults in order to establish a standard to evaluate changed reactions due to disease or drugs.

Electrocardiograms were recorded in one hundred normal adults during and following tilting.

An immediate elevation of blood pressure occurred in all subjects studied during the tilt from the 20 degree erect to the 45 degree head down position. An immediate fall occurred during the return tilt from the 45 degree head down to the 20 degree erect position.

In the normal subjects these primary alterations of blood pressure were followed by a characteristic pattern of depressor response in the head down position and of pressor response in the erect position whereby the blood pressure returned in from eight to eighteen seconds to a level that was approximately the same regardless of the position of the body.

The heart rate invariably slowed in the head down position and increased in the erect position.

In twenty per cent of the normal subjects tilted to and maintained in a head down position the P wave gradually decreased in size and finally disappeared for several beats. The disappearance of the P wave was not associated with a change of the P R interval. This change did not invariably occur in the same individual on successive tiltings.

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## DICUMAROL IN EXPERIMENTAL MYOCARDIAL INFARCTION

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THE first report of the influence of anticoagulants on coronary thrombosis was that of Solandt and Best in 1938<sup>1</sup> who used heparin successfully in experimental animals. Since 1945 many articles have appeared in the medical literature describing the use of Dicumarol for patients with acute myocardial infarction. The first patients to receive anticoagulant<sup>2</sup> therapy were selected because (1) They had suffered repeated episodes of multiple thrombi in different areas of the coronary tree or the original thrombosis had propagated "or (2) "They had suffered repeated embolic phenomena either pulmonary or to other areas." It is apparent that the initial indications were based on analogy with the rationale of anticoagulant therapy in other forms of thromboembolic disease. Because of the beneficial effects of Dicumarol its use was not long restricted to patients with the complications noted; it was given to patients with myocardial infarction as a prophylaxis against such complications. Dicumarol is generally employed in preference to heparin because of the lesser expense and the convenience of the oral route of administration. All who have reported on its use have emphasized the indispensability of accurate prothrombin time determinations to control the dosage.

The incidence of thromboembolic complications in patients with recent myocardial infarction varies in different series of cases. The conflicting data are difficult to analyze and compare since the origin of emboli is seldom determinable with certainty. Hellerstein and Martin<sup>3</sup> found thromboembolic lesions at autopsy in 45 per cent of 160 consecutive cases. Nay and Baines<sup>4</sup> found thromboembolic complications in 37 per cent of 100 consecutive patients with myocardial infarction. Blumer<sup>5</sup> found 16 per cent in his series of 175 patients. Conner and Holt<sup>6</sup> reported 10 per cent in their series of 287 patients and Mintz and Katz observed 9.9 per cent in a series of 572 patients with recent myocardial infarction. These data include not only thromboembolic phenomena arising in the heart but also phlebothrombosis, thrombophlebitis and so forth. The incidence of mural thrombosis of the endocardium is also difficult to ascertain. In autopsy series the reported incidence varies from 17 per cent to 83 per cent.<sup>8</sup> It is evident from these reports that thromboembolism is an important complication of myocardial infarction. In all the reports of the results of anticoagulant therapy there has been an apparently significant reduction in the number of thromboembolic complications and in the general mortality rate. There has been as yet no report which includes suitable controls but such studies are now in progress at a number of centers under the auspices of the American Heart Association. It appears likely that anticoagulants will be used to an increasing extent in the treatment of patients with recent myocardial infarction.

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The chief hazard with anticoagulant therapy is the development of a hemorrhagic state. It is customary to consider any abnormal bleeding tendency, hepatocellular disease, and renal insufficiency as contraindications to the use of Dicumarol. There are no reports in the literature of toxic effects of Dicumarol therapy other than the specific suppression of prothrombin production. Wright<sup>4</sup> mentioned "The possibility that intimal hemorrhage might be a complicating factor (in coronary thrombosis) . . . but no evidence was obtained in this series, either clinically or pathologically, that this was of significance in any case. He also noted that "In no instance was it felt that Dicumarol influenced the rhythm or the rate of the heart directly." It occurred to us that the decreased coagulability of the blood resulting from Dicumarol (or heparin) therapy might affect adversely the course of the myocardial infarct. The early stages of these infarcts are characterized by hyperemia and hemorrhage, which is then followed by necrosis and organization with fibrosis. It seemed possible that the use of anticoagulants could accentuate the hemorrhagic stage of the infarct and thus prolong its resolution. Furthermore, there were no reports in the literature of side effects of Dicumarol which might be advantageous or disadvantageous to the infarcted heart. Such effects might involve facilitation or depression of autonomic reflexes arising in the infarcted myocardium.<sup>5</sup> They might also lead to alterations in conduction leading to changes in the electrocardiogram.

The present study was undertaken to investigate the influence of Dicumarol therapy on the healing of experimental myocardial infarcts. Attention was directed particularly to (1) the mortality rate after the administration of Dicumarol, (2) the extent and character of the infarct, grossly and microscopically, (3) the evolution of the electrocardiographic changes, and (4) the behavior of the sedimentation rate. Anterior infarcts produced by ligation of the anterior descending branch of the left coronary artery were studied because it has been the experience in our laboratory, as well as elsewhere,<sup>6a</sup> that these have a low immediate mortality—less than 25 per cent of the experimental dogs die within twenty-four hours and virtually none die later of cardiac failure. mural thrombosis of the endocardium, or other obvious thromboembolic complications, have not been observed in any of the several hundred dogs with experimental myocardial infarction studied in this laboratory since 1938. On this account it was thought that in dogs any alteration in the mortality rate of experimental myocardial infarction in treated animals would be a primary effect of the Dicumarol, rather than a secondary effect due to the prevention of thromboembolic complications. Since uneventful recovery was the usual finding, deviations from the ordinary course should be easy to recognize.

#### METHODS

Healthy mongrel dogs of either sex were used. Control electrocardiograms were obtained, and blood was drawn for the determination of the prothrombin time, the hematocrit, and the sedimentation rate. Prothrombin time was determined by the method of Quick<sup>10</sup> using a potent rabbit brain thromboplastin. The concentration of the thromboplastin was adjusted so that a value of 6 seconds was obtained with normal dog plasma. This value was assumed to rep-

resent 100 per cent prothrombin time. When longer values for prothrombin time were obtained during Dicumarol therapy they were expressed simply as per cent of the normal value of 6 seconds. This relationship is shown in Table I. In some instances prothrombin time was determined with 12.5 per cent plasma; the average normal value was 16.5 seconds with a range of 13 to 23 seconds. The sedimentation rate was measured in a Wintrobe tube with one reading made at one hour. The hematocrit was determined in the same tube by centrifuging at high speed to constant volume.

TABLE I. RELATIONSHIP BETWEEN PROTHROMBIN TIME IN SECONDS AND PER CENT PROTHROMBIN TIME

PROTHROMBIN TIME (SEC.)	PER CENT PROTHROMBIN TIME
6	100%
7.5	80%
9	67%
12	50%
15	40%
18	33%
21	29%

It is to be noted that per cent prothrombin time represents  $\frac{\text{Normal control prothrombin time}}{\text{observed prothrombin time}} \times 100$ . It does not refer to the dilution of plasma which would give the same number of second clotting time.

One half hour before operation each dog was given a subcutaneous injection of 10 mg. per kilogram morphine sulfate and 0.03 mg. per kilogram atropine sulfate. General anesthesia was produced by the slow intravenous injection of solution of sodium pentobarbital approximately 30 mg. per kilogram. A tracheal catheter was inserted and positive pressure ventilation with oxygen was obtained with a face mask and a water seal. With aseptic precautions the thorax and the pericardial sac were opened from the left side. A ligature was passed around the anterior descending branch of the left coronary artery between its origin and its first major branch. A satisfactory intarct was observed in every instance. Several cubic centimeters of penicillin solution (10,000 units per cubic centimeter) were placed in the pericardial and pleural spaces; the lung was re-inflated and the thorax was closed in layers.

When they regained consciousness the dogs that were to receive Dicumarol were given a first dose of 50 or 100 mg. in a bolus of ground meat. The Dicumarol treated group were given the drug subsequently according to the following schedule:

Prothrombin time less than 15 seconds or more than 40 per cent prothrombin time: 50 mg. Dicumarol.

Prothrombin time from 15 to 25 seconds or between 40 and 24 per cent prothrombin time: 25 mg. Dicumarol.

Prothrombin time more than 25 seconds or less than 24 per cent prothrombin time: no Dicumarol.

Serial determinations of the prothrombin time, the hematocrit and the sedimentation rate and serial electrocardiograms were made on all dogs. Typical sample protocols are shown in Tables IV and V.

The animals were sacrificed at intervals of five to twenty two days after the ligation of the coronary artery. The time of death and the distribution of the groups are shown in Table II. The hearts were removed, cut open, and fixed in Kaiserling I solution. Subsequently the color of the tissues was developed in Kaiserling III solution and kodachrome photographs were obtained. Suitable blocks of tissue were removed for histologic examination.

### RESULTS

Seven of the thirty-two dogs died within twenty-four hours after the production of coronary occlusion, giving an immediate mortality of 21.8 per cent (See Table II). There were no deaths attributable to cardiac failure after the

TABLE II EXPERIMENTAL DATA

Died within 24 hours after coronary occlusion (21.8%)			
Control group			
Sacrificed during first week	2		
Sacrificed during second week	4		
Sacrificed during third week	4		
Total			10
Dicumarol treated group			
Sacrificed during first week	2		
Sacrificed during second week	3		
Sacrificed during third week	8		
Died hemorrhage/infection on eighth and eleventh day respectively	2		
Total			15
Total experimental coronary occlusions			32

first day. Five of the dogs treated with Dicumarol developed complications due to the abnormal hemorrhagic tendency that resulted. Two of these died, apparently from anemia, and the other three became severely anemic. The details of the findings in these animals are shown in Table III. All of the animals but

TABLE III HEMORRHAGIC COMPLICATIONS OF DICUMAROL THERAPY

DOG	DIED (DAY)	SACRIFICED (DAY)	LONGEST IROTTHROMBIN TIME (SEC)	LOWEST HEMA TOCRIT (%)	DICUMAROL (AV DAILY DOSE, MG)	AUTOPSY FINDINGS
8		11	50 (12%)	16	40	Hemothorax
9	13		37 (16%)	15	20	Hemothorax, gastrointestinal hemorrhage
11		16	30 (20%)	15	25	Ukerative colitis, gastrointestinal hemorrhage, hemothorax
14	8		65 (9%)	32*	25	Gastrointestinal hemorrhage
17		21	65 (9%)	10	16	Gastrointestinal hemorrhage

\*This value may represent relative hemoconcentration resulting from the severe diarrhea.

one developed typical myocardial infarcts. There was no mural thrombosis in any animal, and there was no evidence that any thromboembolic phenomena occurred. Typical protocols for a control dog, Dog 16 (Fig 1), and for a dog treated with Dicumarol, Dog 21, are shown in Tables IV and V respectively.

TABLE IV. PROTOCOL DOG 16 BROWN SHORT HAired MALE WEIGHT 11.5 KILOGRAMS

DAY	ECG	SEDIMENTATION RATE	HEMATOCRIT	PROTHROMBIN TIME		DICUMIROL (MG)
				QUICK	PERCENT	
-1	067	13	42	6	14	
0						
1	072		41	6	0	
3	074	15	39	11	11	
6	086	40	40	11	8	
7	--	43	41	11	1	
9	090		--			
10	096	24	41	6	13	
12	102	--				
14	108	24	41	6	11	
17	113	--	--			

Sternfeld

Autopsy There was no evidence of wound infection or pleural effusion in the thorax. There were two ligature imbedded in the anterior wall of the left ventricle. Fibrinous exudate was on the outer surface of the heart which was clearly demarcated infarct of the anterior wall.

TABLE V. PROTOCOL DOG 21 BLACK AND WHITE MALE SHEPHERD WEIGHT 14 KILOGRAMS

DAY	ECG	SEDIMENTATION RATE	HEMATOCRIT	PROTHROMBIN TIME		DICUMIROL (MG)
				QUICK	PERCENT	
-1	106	2	44	6	10	
0						
1	112	8	44	6	10	0
2	--					0
4	117	4	42	12	18	0
6	121	14	6	22	27	--
7	--	--				--
9	127	2	34	4	13	--
10	--	--				--
11	131	--	31	12	30	0
13	137	24	35	0	30	2
14	--	--				--
17	140	20	38	11	32	--
18	--	--				2
18	14	15	40	12	30	0
20	145	11	41	16	38	--
21	149			Sternfeld		

Autopsy The wound was well healed. There was a large pericardial effusion in the wall. There was no effusion. There was a light pericardial effusion in the fluid of which was serosanguineous in character. There was a small area of fibrosis overlying the region of the interventricular septum and some apparent thinning of the apex of the left ventricle. There was a well defined infarct.

The control electrocardiograms were analyzed with respect to the features consistently affected by myocardial infarction. The findings for twenty of the dogs that survived are shown in Table VI. After occlusion of the anterior descending branch of the left coronary artery changes resembling the human  $Q_T$  pattern were observed in fifteen of twenty one animals whose serial electrocardiograms were satisfactory for analysis. An example of the characteristic changes is shown in Fig. 1. The typical features occurred in 57 per cent of the control animals and in 61 per cent of those receiving Dicumilol. See Table VII. It is doubtful that this small difference is significant. The occurrence of changes in  $Q$ ,  $T$ ,  $T-T$ , and ST segments for the entire group (controls and treated) is summarized in Table VIII. The rate of evolution of the  $Q_T$  pattern in each

2510 19211 + 15711 + 2

7-27-77

### Before occlusion

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TABLE VI. ELECTROCARDIOGRAM FINDINGS IN TWENTY CONTROL DOGS

$Q_1$		$T$	
Absent	1	Upright	10
Present	4	Diphasic	3
Deep	1	Isoelectric	0
		Inverted	7
$I_1$		$T$	
Upright	11	Upright	9
Diphasic	1	Diphasic	1
Isoelectric	1	Isoelectric	1
Inverted	7	Inverted	1
ST Segments Any Lead			
No deviation			
Depressed or elevated			

TABLE VII. ANALYSIS OF SERIAL ELECTROCARDIOGRAMS

A. OCCURRENCE OF TYPICAL Q-T PATTERN IN	
Controls	4 out of 8
Dicumarol treated	8 out of 13
Total	12 out of 21
B. OCCURRENCE OF ARRHYTHMIAS	
Premature ventricular contractions in	
Controls	6 out of 8
Dicumarol treated	13 out of 13
Premature nodal contractions and bundle branch block in	
Controls	0 out of 8
Dicumarol treated	4 out of 13

TABLE VIII. EFFECT OF ANTERIOR INFARCTION ON Q-T PATTERN IN SERIAL ELECTROCARDIOGRAMS, TWENTY ONE SATISFACTORY RECORDS

$Q$		$T$	
No change	7	No change	1
Control to deep	13	Upright to inverted	1
Deep to absent	1	Upright to inverted to upright	9
		Inverted to upright	7
$T$		$T$	
No change	10	No change	14
Upright to inverted	7	Upright to inverted	4
Upright to inverted to upright	2	Upright to inverted to upright	1
Inverted to upright	2	Inverted to upright	1
ST Segment			
Reciprocal displacement Lead I and III		11	
No reciprocal displacement		10	

group is summarized in Table IX. There was no difference in the rate of evolution of the changes in  $Q_1$ . In the animals that received Dicumarol inverted  $T_1$  returned to normal on an average of one day later than in the control dogs. The significance of this difference also is doubtful. Arrhythmias (see Table VII B) were found more frequently in the electrocardiograms of the dogs that received Dicumarol. However it is our experience that when electrocardiograms are made daily in dogs, very few fail to show some arrhythmia after coronary occlusion. In the present experiment, tracings were not always made daily

during the first week when the transitory arrhythmias occurred. Bundle branch block occurred twice in the treated group, but this is not infrequent as a transient occurrence in dogs with myocardial infarction.

TABLE IX. EVOLUTION OF  $Q_1$ ,  $T_1$  PATTERN IN SEVENTEEN ANIMALS SACRIFICED IN SECOND AND THIRD WEEK AFTER CORONARY OCCLUSION

$Q_1$	
Controls (6)	Five developed deep $Q_1$ One had no significant change in $Q_1$ One returned to normal on twenty-first day Four were sacrificed before change occurred
Dicumarol treated (11)	Six developed deep $Q_1$ Five had no significant change in $Q_1$ Five were sacrificed before change occurred One returned to normal on fourteenth day
$T_1$	
Controls (6)	Five developed inverted $T_1$ Five returned to normal by eighth day One had no significant change in $T_1$
Dicumarol treated (11)	Five developed inverted $T_1$ Four became normal by ninth day One died on eighth day $T_1$ inverted Six had no significant change in $T_1$

With one exception all the hearts removed at autopsy were found to have infarcts of the apical and anterior septal regions, with myocardial, subepicardial and subendocardial hemorrhage. The sole exception, Dog 19 a control, had no visible infarct. The ligature was in place and the coronary artery was occluded. Large anastomotic branches were easily seen connecting the occluded branch with the circumflex branch of the left coronary artery. There did not appear to be any significant, consistent difference in the size of the infarcts or the amount of hemorrhagic infiltration in the two groups of animals. This judgment is based on experience with more than 100 dogs' hearts with anterior infarction. Reproductions of kodachrome photographs of the control and the treated hearts sacrificed at similar times are shown in Figs. 2, 3, and 4. Pleural and pericardial adhesions occurred with equal frequency in each group of dogs. It is worthy of comment that the extent of these adhesions was much less than in our previous experience. The difference is most certainly due to the action of penicillin, applied topically, in preventing infection. Microscopic examination of representative sections was performed by Dr. William B. Waitman.\* It was his opinion that there was no obvious difference between the healing in infarcts in the treated animals and the controls.

Using the Quick method for prothrombin time and following the schedule of dosage of Dicumarol noted previously, it was possible to regulate the clotting activity of the blood in a satisfactory manner. Excessive dosage and the appearance of abnormally high values for prothrombin time occurred over holidays and week-ends when the usual daily dose was continued without laboratory control.

\*Professor of Pathology, Northwestern Medical School.



Fig. 2—Right Dog 3 control sacrificed on the fifth day. Left Dog 4 control sacrificed on the fifth day received a total of 40 mg. Dicumarol maximum value for prothrombin time was 1 second. There is very little difference in the appearance of the two hearts.

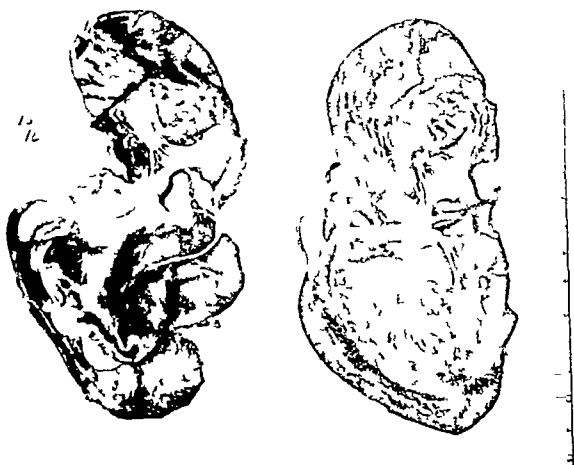


Fig. 3—Right Dog 1 sacrificed on the seventeenth day received a total of 40 mg. Dicumarol maximum value for prothrombin time was 31 seconds. Left Dog 10 control sacrificed on the seventh day. There is no difference in the appearance of the two hearts.

In every instance in which abnormal bleeding occurred, the prothrombin time was longer than 25 seconds. In the control group the prothrombin time was shorter than the lower limit of normal during the first week after occlusion in four of the seven animals with satisfactory serial tests. (See Table IV) Increased clotting activity of these dogs was apparent only when 125 per cent



Fig. 1—Right: Dog 17, sacrificed on the twenty-first day, received a total of 300 mg. Dicumarol; the maximum value for prothrombin time was 65 seconds. Left: Dog 11, sacrificed on the sixteenth day, received a total of 125 mg. Dicumarol; the maximum value for prothrombin time was 30 seconds.

plasma was used for the determination. The variations in the sedimentation rate were not consistent and were of no importance in evaluating the course of experimental myocardial infarction. The hematocrit was a useful index of the extent of such anemia as developed.

#### SUMMARY

Myocardial infarction was produced in twenty-five dogs by ligation of the anterior descending branch of the left coronary artery. Fifteen of the animals were given Dicumarol in amounts equivalent to those used in the management of patients with recent myocardial infarction. There was no evidence that the altered coagulability of the blood affected the extent or the healing of the infarcts. Serial electrocardiograms did not show any consistent significant difference between the treated animals and the controls.

## CONCLUSION

The use of the anticoagulant Dicumarol does not have any demonstrable deleterious influence on the healing of experimental myocardial infarction in dogs.

The authors wish to express their gratitude to Miss Ruth A. Trump, B.Sc., for her painstaking laboratory work.

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# THE EFFECTS OF DICUMAROL ON THE ELECTROCARDIOGRAM

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THROMBOEMBOLIC complications long have been recognized as a major hazard in the course of acute myocardial infarction<sup>1,2</sup> Increased blood clotting tendency following myocardial infarction has been demonstrated repeatedly.<sup>4,5</sup> Anticoagulant therapy with Dicumarol has been invoked accordingly by many clinicians as a prophylactic measure to neutralize the increased propensity to thromboembolism following myocardial infarction. In the past several years sufficient experience with this drug has accumulated to show substantial (in some reports even remarkable) reduction in thromboembolic catastrophes.<sup>7,8</sup> Our own favorable experience thus far has been sufficiently persuasive to justify addition of controlled Dicumarol therapy to the routine regimen for every patient with acute myocardial infarction.

There are scattered references in the literature to the relative harmlessness of Dicumarol so far as the heart is concerned, Blumgart and co-workers showed that in dogs whose coronary arteries had been ligated Dicumarol had no adverse effects on the evolution of the infarction *per se*.<sup>5,6</sup> In a field where electrocardiographic developments are depended on for evaluation of myocardial change it becomes important to determine whether any of the electrocardiographic changes are due to the drug taken. The purpose of this study was to establish whether Dicumarol *per se* has any effects on the electrocardiogram, and, if so, what these effects are.

## METHODS

Dicumarol was given to forty-eight subjects. Of these, twelve were normal, with no discoverable evidence of cardiovascular disease and with repeatedly normal electrocardiograms (Group 1). Sixteen were patients with varying types of cardiovascular disease and a variety of abnormal electrocardiographic patterns which remained stable during preliminary observation (Group 2). Daily electrocardiograms were taken at each step of the progressive fall in the prothrombin level until prothrombin activity between 15 and 25 per cent of normal was attained. In several instances Dicumarol dosage was deliberately increased until prothrombin concentration fell to less than 10 per cent of normal so as to observe the effects of such dosage on the electrocardiogram. Inasmuch as in our cases of acute myocardial infarction anticoagulant therapy is continued until the patient becomes ambulatory, the effect of adequate Dicumarol medication continued for at least four weeks was observed in five of the normal subjects and in four of the subjects with cardiovascular disease (Group 1 and Group 2, respectively).

In addition we studied the serial electrocardiograms in fourteen of our patients with acute infarction who were on a Dicumarol regimen (Group 3) for evidence of possible Dicumarol effects, both during the period of Dicumarol treatment and following withdrawal of the drug.

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A separate group of six digitalized patients (Group 4) whose electrocardiograms had remained stable under prolonged observation were also treated with Dicumarol in the manner described to observe the possible modifying influence of the drug on the overall degrees of digitalis effect.

In all subjects a preliminary study of renal function was done and a pretreatment prothrombin level was obtained. In the course of Dicumarol administration the drug effect was closely controlled with daily prothrombin level determination and toxicity was watched for by daily study for microscopic hematuria, examination of the skin for hemorrhagic extravasations, and inquiry about bleeding from gums, nose and gastrointestinal tract.

Aside from the maintenance dose of digitalis received by the six patients of Group 4 the only medication given was an occasional dose of aspirin and in Group 3 aspirin and papaverine during the early stages of acute myocardial infarction.

Leads I, II, III, CF<sub>1</sub>, CF<sub>2</sub> and CF were taken in all instances. Electrocardiograms were analyzed with respect to rhythm, auriculoventricular and intraventricular conduction, Q-T duration, R-S-T level, and amplitude of auricular and ventricular components.

The Quick method was used to determine prothrombin activity. Each batch of thromboplastin substance was calibrated against a number of normal sera in several dilutions to minimize the errors of interpolation. Results were expressed as a percentage of normal prothrombin activity.

## RESULTS

In Groups 1 and 2 all electrocardiograms taken at successively lower prothrombin levels were substantially the same. An occasional tracing showed minimal changes in amplitude of T<sub>1</sub> and more rarely in amplitude of P commensurate with the associated changes in rate. In the precordial leads minor changes in R/S ratio were seen attributable to slight shifting of the exploring electrode during successive tracings.

In those subjects maintained for several weeks on adequate doses of Dicumarol no appreciable change appeared in the electrocardiograms as a result of the prolonged medication.

Subjects given maximal doses of Dicumarol with depression of prothrombin to less than 10 per cent of normal showed no electrocardiographic effects of their hypoprothrombinemia.

The serial electrocardiograms of patients with acute myocardial infarction (Group 3) showed the expected progressive changes of healing infarction. The rate of progress did not differ materially from that observed in patients not receiving Dicumarol. Withdrawal of the drug during convalescence did not cause significant electrocardiographic change in any of these cases. The residual electrocardiographic abnormalities in this small series fell within the expected range of late postinfarction patterns.

All of the group of patients whose electrocardiograms showed abnormalities attributable to digitalis (Group 4) retained the respective patterns unchanged by effective doses of Dicumarol and the electrocardiograms remained stable after withdrawal of the Dicumarol. In one of these patients occasional ventricular extrasystoles appeared in the course of the study and disappeared again when digitalis was withheld for two days. Dosage of Dicumarol was not changed during this interval and the electrocardiogram was not otherwise affected.

Questionable toxic effects were observed in only one of the subjects. On a morning when the prothrombin concentration was 15 per cent of normal the

patient reported coughing up (or possibly vomiting) a small quantity of blood streaked material. There was no associated evidence of bleeding in the skin, mucous membranes, or urine, and no change in blood pressure, heart rate, or red cell count. The electrocardiogram that day did not differ from previous and succeeding tracings. The patient was given 20 mg of synkavite intravenously, and Dicumarol was withheld. There were no further adverse manifestations.

#### SUMMARY

The addition of Dicumarol to the therapeutic regimen of patients with acute myocardial infarction suggested the investigation of the effects of the drug on the electrocardiogram.

The subjects of this study, forty-eight patients with and without heart disease and with a wide variety of electrocardiographic patterns, were maintained for a period on adequate doses of Dicumarol. Serial electrocardiograms taken during this period were analyzed.

No significant electrocardiographic deviation attributable to Dicumarol was observed in any of the subjects.

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# THE LIFE SPAN OF THE SICKLE CELL AND THE PATHOGENESIS OF SICKLE CELL ANEMIA

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THE mechanism responsible for the development of sickle cell anemia is not yet understood. Only relatively few Negroes who harbor the sickle cell trait are afflicted with this hemolytic syndrome. Recent *in vitro* studies of the sickling phenomenon in our laboratory<sup>1</sup> failed to demonstrate any fundamental difference between trait and anemia cells. When susceptible erythrocytes are suspended in a culture of *Bacillus subtilis* either sickling or oat cell formation occurs regularly after from five to fifteen minutes.<sup>1</sup> No distinct correlation could be detected between the number of sickle cells in the preparation or the rapidity and degree of the sickling process and the presence of either trait or anemia. Therefore it seemed conceivable that an additional qualitatively different factor might be operating in the production of the anemia.

As has been pointed out in a previous paper<sup>2</sup> the various hemolytic syndromes may quite generally be classified in two main groups. The first comprises all the disorders in which red cells are damaged by an extracorporeal mechanism (for example malaria). In the disorders belonging to the second group, premature disintegration of the erythrocytes is apparently caused by a primary abnormality of the stroma which may manifest itself morphologically (for example familial hemolytic jaundice). This classification is based on the so-called cross determination of the survival time of red cells. When normal erythrocytes transfused into a recipient with a hemolytic syndrome survive normally whereas the recipient's own red cells transfused into a normal person have a considerably shortened life span, an intracorporeal anomaly may be suspected. Conversely when normal red cells transfused into the patient with the hemolytic syndrome are as rapidly destroyed as the patient's own cells the presence of an extracorporeal mechanism may be assumed.

This paper deals with cross determinations of the survival time of sickle cells. Trait cells were transfused into patients with sickle cell anemia and anemia cells into healthy recipients displaying the sickle cell trait.

## MATERIAL AND METHODS

The diagnosis of sickle cell anemia in patients used in this study was based on the presence of a marked or moderate anemia, increase of the reticulocyte count, and elevation of the serum bilirubin, besides a compatible clinical picture.

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ture and a positive sickle cell test. Almost all individuals considered to represent instances of the sickle cell trait had entirely normal hematologic findings except for the positive sickle cell test. Each patient had at least two complete hematologic examinations before being chosen either as donor or as recipient.

*Hematologic Determinations*—Five cubic centimeters of blood were obtained by venipuncture and transferred to a vial containing 4 mg potassium oxalate and 6 mg ammonium oxalate. From this oxalated blood the following determinations were regularly performed:

*Hemoglobin* Photoelectric method of Sheard and Sanford<sup>3</sup> (Evelyn colorimeter), 100 per cent equals 15.6 Gm per 100 cc of blood.

*Red Cell and White Cell Counts* United States Bureau of Standards certified pipettes and hemocytometer used. Automatic shaker.

*Hematocrit* Centrifugation in Wintrobe type of hematocrit tubes for thirty minutes at 3,500 revolutions per minute.

*Bilirubin in the Plasma* Method of Malloy and Evelyn.<sup>4</sup>

*Sickle Cell Test* The rapid method of Singer and Robin.<sup>1</sup>

Furthermore, smears were prepared from capillary blood on cover slips and stained with Wright's stain. The reticulocyte count was obtained by the dry method.

*Methods Used for the Determination of the Survival Time*—Determinations of the survival time were done with the method of differential agglutination (Ashby technique). Either O cells were transfused into recipients belonging to group A or B, or, if donor and recipient were of the same group, the donor's cells containing the N agglutininogen were introduced into an M or MN recipient. Anti-M serum was then employed for agglutinating out the recipient's own cells in the follow-up studies. Anti-N serum was used only for typing and not for any quantitative evaluation on account of its known unreliability for this purpose.<sup>5</sup>

Typing for the Landsteiner blood groups was performed with potent sera prepared by the Serum Center.<sup>\*</sup> Testing for the M and N factors was done with sera supplied by the Certified Blood Donor Center.<sup>†</sup> In all patients the Rh factor was determined by means of a human anti Rh serum of high titer.

Blood for transfusions was obtained by venipuncture and was transferred into a flask containing 50 cc of a modified a c d mixture. Then the plasma was removed and replaced by a sufficient amount of a dextrose saline solution.<sup>‡</sup>

An adequate replacement transfusion was given to all anemic donors immediately after the bloodletting, thus more than compensating for the clinically undesirable loss of erythrocytes.

For the follow-up study of the transfused cells, the method of Young, Platzer, and Rafferty<sup>6</sup> was found to be very satisfactory. Each recipient had at least two determinations of the nonagglutinable cells. Since very potent sera were used, the number of nonagglutinable cells never exceeded 50,000 per cubic millimeter.

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<sup>‡</sup>We are indebted to Dr. S. Levinson and Dr. A. Wolf of the Serum Center, Michael Reese Research Foundation, for their invaluable assistance in the processing of the erythrocytes.

Only young children (1 to 6 years old) were selected as recipients. Because of their small circulating blood volume a correspondingly small transfusion was found sufficient to result in a satisfactorily high addition of the donor's cells per cubic millimeter. Packed cells derived from 200 to 500 cc of whole blood were introduced, yielding an initial increase of 540 000 to 2 million cells per cubic millimeter in the recipient's total erythrocyte level. These initial values were established twenty-four hours after transfusion in order to avoid any significant inaccuracies due to an augmentation of the circulating fluid volume<sup>7</sup> although it is realized that some of the transfused cells already may have been eliminated within the first twenty-four hours. After determining the starting points, follow-up studies were done twice weekly during the first month and from then on every week.

### RESULTS

*Transfusion of Trait Cells Into Patients With Sickle Cell Anemia*—Experiments of this type were performed in three instances. The hematologic data of the respective donors and recipients are compiled in Table I. Fig. 1

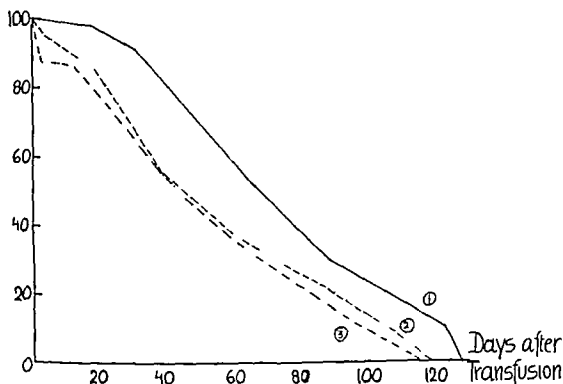


Fig. 1—Transfusion of sickle cell trait cells into patient with sickle cell anemia expressed in per cent of surviving donor's cells

shows the curves illustrating the disappearance of the donor's cells. The percentage of the surviving cells is plotted against the number of days following transfusion. In all three instances the survival time of the trait cells was normal, with an average of 120 days. Thus trait cells when transfused into patients with sickle cell anemia have a normal survival time.

Fig. 2 shows the various red cell counts of one of the transfused recipients. These values represent the sum total of two populations of erythrocytes: that is, the recipient's own cells and the donor's cells. As can be seen from the illustrations, the patient's own cells were first hemolyzed much more rapidly than the transfused trait cells. After about thirty days there was an increase of the

TABLE I TRANSFUSION OF

EXPERIMENT	DONOR								
	AGE (YR)	SEX	HGB		RBC (MILLION)	WBC (1,000)	RETIC (%)	BILI RUBIN (MG %)	TYPING FORMULA
			GM	%					
1	50	F	12.1	78	4.90	5.9	0.8	0.4	A Rh <sub>+</sub> N
2	30	F	12.7	82	4.28	5.1	0.2	0.56	O Rh <sub>+</sub>
3	28	F	13.0	84	5.08	12.2	1.0	0.4	O Rh <sub>+</sub>

patient's own cells. When the transfused cells had completely disappeared, approximately the same degree of anemia was present as existed before the transfusion. Similar results were obtained from an analysis of the total counts of the two other patients in this group.

*Transfusion of Sickle Cell Anemia Cells Into Recipients With Sickle Cell Trait*—Red cells from four patients with sickle cell anemia were transfused into three patients with sickle cell trait. In one experiment the red cells from two anemic patients both having blood group O were mixed and introduced into a single recipient, thus creating three different populations of red cells in this recipient. The hematologic data of the individuals used in this second group of studies may be found in Table II. Recipient 1 was a patient with non deficiency anemia harboring the sickle cell trait. This patient had a hypochromic microcytic anemia with absence of target cells and showed a typical reticulocyte response with elevation of hemoglobin and erythrocytes after administration of iron.

TABLE II TRANSFUSION OF

EXPERIMENT	DONOR									
	AGE (YR)	SEX	HGB		RBC (MILLION)	WBC (1,000)	RETIC (%)	BILI RUBIN (MG %)	TYPING FORMULA	VOLUME OF BLOOD USED FOR TRANSFUSION (CC)
			GM	%						
1	8	M	7.9	51	2.92	5.4	11.5	1.6	O Rh <sub>+</sub>	220
2	5	M	7.8	50	2.39	22.4	8.0	1.9	B Rh <sub>+</sub> N	2200
3a	12	M	7.5	48	2.48	18.3	13.4	2.4	O Rh <sub>+</sub>	200
3b	6	F	9.7	62	3.65	14.9	4.4	0.7	O Rh <sub>+</sub>	200

## UTS WITH SICKLE CELL ANEMIA

RECIPIENT								RESULTS		
C	HB		R.B.C (MILLION)	W.B.C (1000)	PCTIC (%)	BILI RUBIN (MG %)	TYING FORMULA	REMARKS	INCREASE OF INITIAL R.B.C IN RECIPIENT AFTER TRANS FUSION (MILLION)	SUR VIVAL TIME (DAYS)
	GM	%								
	0.2	40	2.1	197	16.0	2.5	A Rh+ M	Numerous target cells	2.16	127
	9.62	4.03	97	5.0	1.2	A Rh+		Numerous target cells	1.54	118
	9.51	3.33	10.0	16.6	2.4	B Rh+		Numerous target cells 10 Nucleated R.B.C per 100 W.B.C	2.01	114
									Average	120

In all instances there was a definite shortening of the survival time\* of the anemia cells (Fig. 3). In the first two experiments the anemia cells were no longer demonstrable after thirty and thirteen days respectively. Of special interest is the profile of Curve 3. At first 46 per cent of the introduced cells disappeared rapidly within fourteen days; then there was a lag of ten days which was followed by a further but conspicuously slower decrease of the number of transfused cells. All anemia cells had disappeared after fifty-eight days. Although no proof can be offered, one may interpret this stepped line of decay as being caused by an initial very rapid elimination of the red cells of Donor 3a who had a very active hemolytic process. Then later the remainder of the cells, mostly originating from the second donor (Donor 3b) with a much milder hemolytic syndrome, disappeared more slowly. However, the number of cases

While these experiments were in progress Callender and Nickel<sup>8</sup> read a preliminary paper at the Twentieth Annual Meeting of the Central Society for Clinical Research on survival time determinations of sickle cell anemia cell in normal recipients. Their results concur with ours. Since their studies also were not completed at that time their final results and interpretations cannot be taken into consideration here.

## ELS INTO SICKLE CELL TRAIT CARRIERS

RECIPIENT							RESULTS		
HB		RBC (MILLION)	WBC (1000)	RETIC (%)	BH1 RUBIN (MG %)	TYING FORMULA	REMARKS	INCREASE OF INITIAL RBC IN RECIPIENT AFTER TRANS- FUSION	SURVIVAL TIME (DAYS)
GM	%								
815		548	105	40	04	B Rh+	Iron deficiency anemia with sickle cell trait	441 000	30
13889		486	91	04	05	B Rh+ M		545 000	13
10178		413	15	04	03	B Rh+		1130 000	38
								Average	34

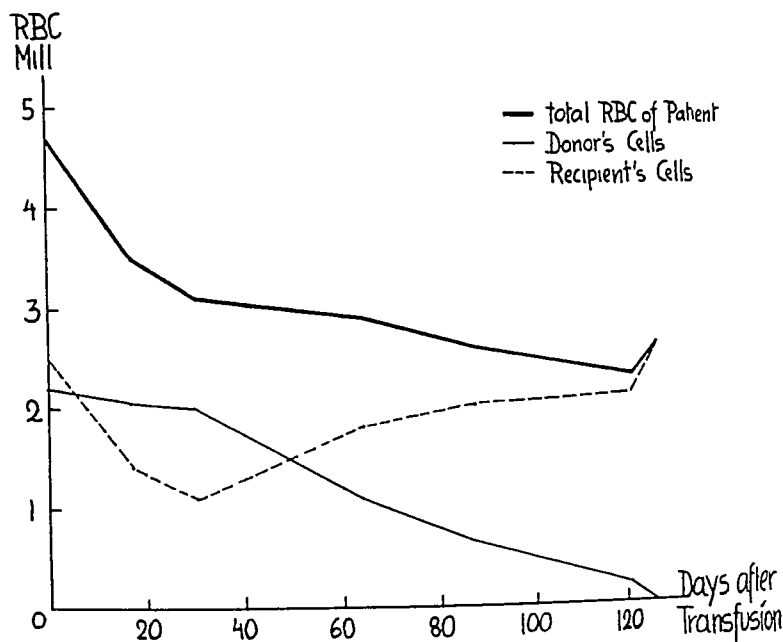


FIG 2—Transfusion of sickle cell trait cells into patient with sickle cell anemia

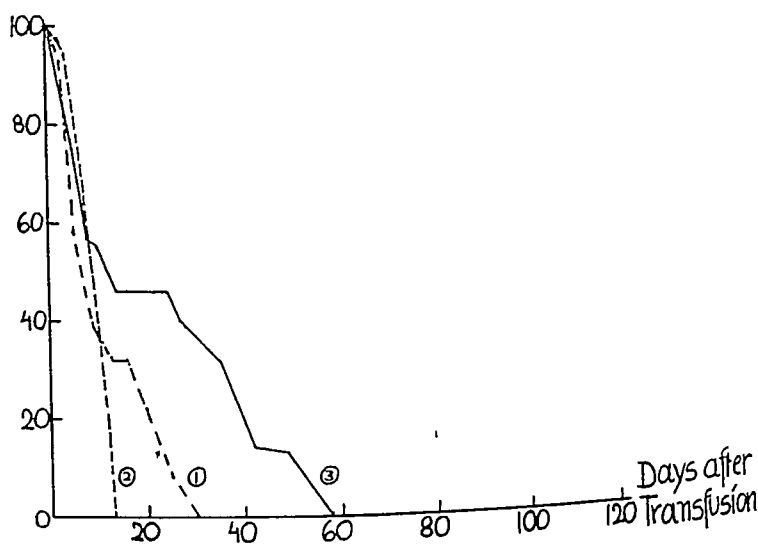


FIG 3—Transfusion of sickle cell anemia cells into recipients with sickle cell trait expressed in per cent of surviving donor's cells

is too small to establish any definite correlation between the clinical severity of the donor's hemolytic process and the survival time. This problem requires further study, particularly in cases during hemolytic crisis.

## DISCUSSION

The average life span of the red cell in dogs and human beings under physiologic conditions is approximately 120 days. This now must be considered as an established fact. By quite different methods for instance pigment studies in dogs with renal biliary fistula<sup>9, 11</sup> and survival time determination after transfusion of variously labeled red cells (Ashby technique<sup>23</sup> sulfhemoglobin,<sup>11</sup> radioactive mitogen<sup>12</sup>) others have arrived at this same figure. British workers,<sup>13, 14</sup> using the method of differential agglutination plotted the numbers of surviving cells against time in a diagram and found a linear type of disappearance. Normally a fixed fraction of those cells introduced at transfusion are removed in the unit of time (0.53 per cent per day). This straight line of decay depends on a property (age) of the transfused cells themselves. The finding of such a straight line of disappearance over a period of approximately 120 days may therefore be interpreted as the expression of erythrocytic disappearance by normal means.

If abnormal conditions exist these two criteria are altered: the survival time is considerably shortened and the graphs are either straight but steep or show a curved appearance. Such abnormal lines of decay are indicative of the presence of abnormal hemolytic mechanisms. By means of the cross determination of the survival time one may then gain better insight into the nature of these pathologic conditions. When normal erythrocytes transfused into a recipient with a hemolytic syndrome survive normally, whereas the recipient's own cells transfused into a normal person have a considerably shortened life span an intracorporeal abnormality may be suspected. Contrariwise when normal red cells transfused into a patient with a hemolytic syndrome are as rapidly eliminated as the patient's own cells an indiscriminating extracorporeal mechanism can be assumed.

Our experiments demonstrate that trait cells have a normal survival time and an almost straight line of decay when introduced into patients with sickle cell anemia. Anemia cells however when transfused into trait carriers are destroyed much more rapidly as can be seen from the abnormal graphs. Therefore the pathogenic principle operating in sickle cell anemia resides within the red cells themselves; no extracorporeal mechanism can be made responsible for this hemolytic syndrome. Altman<sup>15</sup> and Callender and Nickel<sup>8</sup> reported that the longevity of normal erythrocytes in patients with sickle cell anemia is unaltered. The finding that trait cells also survive normally is of practical importance because these erythrocytes can be used safely for therapeutic transfusions.

Our investigations have established a fundamental difference between the survival time of anemia and trait cells. Since both types of cells show the sickling tendency the question arises whether this difference is related to a variable degree of the basic abnormality responsible for the sickling process or whether an additional structural alteration of the anemia cell may be involved. The following facts and observations require consideration in the evaluation of this problem.

(1) The well-known clinical fact that one does not encounter transitional types between sickle cell anemia and sickle cell anemia cases would support the existence of an additional pathogenic factor in the production of the anemia

(2) Hahn and Gillespie,<sup>16</sup> in 1927, made the significant discovery that distortion of susceptible corpuscles into sickle cells will take place only if the hemoglobin contained in the cells is in the reduced state. In vitro studies of the sickle cell phenomenon fail to show any clear distinction between trait and anemia if the sickling test is performed under conditions optimal for the reduction of hemoglobin. Neither with the gas chamber method of Hahn and Gillespie<sup>16</sup> nor with the rapid method of Singer and Robin,<sup>1</sup> who used a broth culture of *B. subtilis* for the reduction of the oxyhemoglobin, could any definite correlation be detected between the number of sickling cells in the preparation or the rapidity and degree of the sickling process and the presence of either trait or anemia. The often demonstrable difference in the results between patients with anemic and trait cells with the moist stasis method<sup>1</sup> is probably caused by the increased number of nucleated red cells, reticulocytes, and white cells present in the anemia blood, only these cells consume oxygen, whereas mature red cells do not. As a proof of this interpretation, the observation<sup>1</sup> may be offered that when trait cells are mixed with the buffy layer of leucemic blood, sickling can almost immediately be elicited, although the unmixed blood may require more than twenty-four hours with the stasis method to achieve this result. On the other hand, intravascular sickling has been observed in anemic patients much more commonly than in trait carriers,<sup>18</sup> and it has been speculated that blood destruction may be due to vascular stasis and obstruction of the capillaries by sickling red cells. Such a pathogenic mechanism, however, is unlikely because it would not account for the demonstrably different survival times of trait and anemia cells in the same patient.

(3) The experiments of Reinhard and co-workers<sup>19</sup> also seem to retute the concept that a more pronounced sickling of cells in vivo may be instrumental in causing the anemia. These workers induced patients with sickle cell anemia to breathe high oxygen concentrations for periods of from eight to twenty days without detecting any inhibition of the rate of hemolysis, although a considerable decrease in the degree of intravascular sickling was noted.

(4) The finding that the pathogenic principle of sickle cell anemia resides within the red cells may lend itself to an explanation of the well known hemolytic crises which occur in this disorder. Murphy and Shapiro<sup>20</sup> suggested that the age of the red cell exerts an influence on its sickling tendency. According to these authors, normoblasts and reticulocytes are rarely seen in sickle form. This latter statement is not corroborated by our observations when optimal conditions for eliciting the sickling process are maintained. However, one may speculate whether the alterations of the stroma which occur with aging when superimposed on an already structurally defective cell, do not really lead to the massive disintegration of erythrocytes in a short period of time which is the outstanding hematologic feature of the crisis. After such an episode, the rejuvenation of the blood by many reticulocytes takes place. The



the same process repeats itself in relation to the average survival time of the cells in the circulation. This concept could be tested by correlating the time intervals between crises with the life spans of the red cells before and after such hemolytic exacerbations.

As can be seen from this discussion the known facts about the sickling phenomenon do not readily explain the pathogenesis of the anemia. Sickling is the expression of an abnormality of the stroma.<sup>1</sup> If together with this abnormality there exists an additional alteration in the cytoskeleton<sup>22</sup> the cell structure becomes much more vulnerable to the vicissitudes of life in the circulation. Thus the survival time of the anemia cell is shortened. We are fully aware that this hypothesis is not yet based on any positive evidence. New experiments must be devised to furnish more factual support for this concept.

#### SUMMARY

The method of cross determination of the survival time of red cells was used for the study of the pathogenesis of sickle cell anemia. When normal erythrocytes transfused into a recipient with a hemolytic syndrome survive normally, whereas the recipient's own red cells transfused into a normal person have a considerably shortened life span, an intracorporeal abnormality may be suspected. Contrariwise, when normal red cells transfused into the patient with the hemolytic syndrome are rapidly destroyed as the patient's own cells, the presence of an indiscriminating extracorporeal mechanism may be assumed. In this study, trait cells were transfused into patients with sickle cell anemia and anemia cells into healthy recipients harboring the sickle cell trait.

Trait cells survive normally (120 days) when transfused into patients with sickle cell anemia, whereas the patient's own cells continue to be hemolyzed at a faster rate. Anemia cells when transfused into trait carriers have a shortened life span with an average of about one fourth of the normal. Therefore the pathogenic principle operating in sickle cell anemia resides within the red cells themselves; no extracorporeal mechanism can apparently be made responsible for this hemolytic syndrome.

Trait cells can be used safely for therapeutic transfusions.

An analysis of the known facts about the sickling phenomenon shows that the sickling process, which is the expression of an abnormality of the stroma, does not lend itself to a satisfactory explanation of the pathogenesis of the anemia. The hypothesis is formulated that sickle cell anemia develops because of an additional alteration in the cytoskeleton which is qualitatively different from the structural anomaly responsible for the sickling phenomenon.

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# INTRAGROUP INCOMPATIBILITY WITH RESPECT TO THE H<sub>1</sub> BLOOD FACTORS AS A CAUSE OF MINOR HEMOLYTIC TRANSFUSION REACTIONS

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THE work of Wiener and Peters<sup>1</sup> in demonstrating the role of the Rh factor of Landsteiner and Wiener<sup>2,3</sup> in the pathogenesis of intragroup hemolytic transfusion reactions now has been amply confirmed.<sup>4-11</sup> This has brought about an important change in the practice of blood transfusion namely the inclusion of routine Rh testing along with A B blood grouping tests as a basis for the selection of donors for blood transfusion. The proper application of these tests has led to the virtual elimination of dangerous or major hemolytic reactions as a complication of transfusion. It gradually has become recognized moreover that Rh positive individuals also can be isosensitized because of the existence of more than one variety of Rh factor and the so called H<sub>1</sub> factors.<sup>12-14</sup> Unlike these other blood factors are far less antigenic than the original rhesus factor (Rh<sub>0</sub>) of Landsteiner and Wiener, so that such cases are quite rare. When isosensitization does occur it hardly ever reaches the degree characteristic of Rh or A B sensitization so that the antibodies usually are not demonstrable in the sensitized individual's serum and the reactions following transfusions of incompatible blood are mild and simulate pyrogenic reactions.<sup>1</sup> These minor hemolytic reactions occurring in Rh positive recipients are the subject of the present paper.

Paralleling the improvements in transfusion practice brought about by the discovery of the rhesus blood factors there also has been progress in eliminating pyrogens as a cause of nonspecific chills and fever following blood transfusions. For example at the Jewish Hospital of Brooklyn during the period 1945 to 1947 the total number of blood transfusions carried out annually increased from 1800 to 3800 yet the number of untoward reactions decreased from fifty eight to forty seven a drop in rate of reactions from 2.6 to 1.2 per cent. This is shown in Table I, together with the figures during a comparable period a decade ago.<sup>\*</sup> The virtual elimination of pyrogenic reactions by meticulous care in preparing the blood transfusion apparatus together with the prevention of major hemolytic reactions by improvements in blood typing techniques, has served to bring to the fore the other class of reactions namely the minor hemolytic reactions which form the subject of this report.

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As will be seen from the Table I at the present time the bulk of the blood used at the Jewish Hospital of Brooklyn is obtained from the Blood and Plasma Exchange Bank of New York City so that this improvement is to be credited primarily to the admirable work of Dr. Lester J. Unger who is director of that blood bank which provides many thousand units of blood every month to hospitals throughout the country.

TABLE I COMPARISON OF INCIDENCE OF TRANSFUSION REACTIONS TODAY AND TEN YEARS AGO

YEAR	TOTAL NUMBER OF TRANS- FUSIONS	BLOOD USED (UNITS)	TECHNIQUE OF TRANSFUSION			INCIDENCE OF REACTIONS	
			UNMODIFIED BLOOD (SYRINGE VALVE)	FRESH CITRATED BLOOD	BANK BLOOD	NUMBER	PER CENT
1936-37 <sup>4</sup>	1,209	—	950	259	—	95	7.9
1938*	976	—	360	616	—	22	2.2
1939*	1,213	—	267	946	—	35	2.9
1945	1,884	2,121	2	481	1,638	58	3.1
1946	2,496	2,970	—	581	2,452	50	2.0
1947	3,872	3,978	—	613	3,365	47	1.2

\*These data are taken from the paper of Wiener A S, Oremland B H, Hyman M A and Samwick A A. Transfusion Reactions: Experiences With More Than 3,000 Blood Transfusions. *Am J Clin Path* 11: 102-121 1941.

THE Rh-H<sub>1</sub> BLOOD TYPES

In early studies on human anti-Rh sera it was soon found that in addition to the type corresponding to the original anti-rhesus serum (now designated anti-Rh<sub>0</sub>),<sup>2, 3</sup> which gave 85 per cent positive reactions on Caucasian blood, two other varieties of anti-Rh exist, namely anti-1h' (70 per cent positive)<sup>2, 4</sup> and anti-1h'' (30 per cent positive)<sup>10, 17</sup>. The three anti-Rh sera determine three corresponding factors Rh<sub>0</sub>, 1h', and 1h'' in human blood, which in combination give rise to eight types of blood instead of two, Rh positive and Rh negative.<sup>18</sup>

TABLE II SCHEME OF THE EIGHT Rh BLOOD TYPES

BLOOD CONTAINING FACTOR Rh <sub>0</sub> (Rh POSITIVE)				BLOOD NOT CONTAINING FACTOR Rh <sub>0</sub> (Rh NEGATIVE)			
DESIGNATION OF TYPES*	REACTIONS WITH SERA			DESIGNATION OF TYPES*	REACTIONS WITH SERA		
	ANTI 1h'	ANTI 1h''	ANTI Rh <sub>0</sub>		ANTI rh'	ANTI rh''	ANTI Rh <sub>0</sub>
Rh <sub>0</sub>	—	—	+	rh	—	—	—
Rh <sub>1</sub>	+	—	+	1h'	+	—	—
Rh	—	+	+	rh''	—	+	—
Rh <sub>1</sub> Rh	+	+	+	1h'rh''	+	+	—

\*Type Rh<sub>1</sub> contains the two factors Rh<sub>0</sub> and 1h', the name Rh<sub>1</sub> being short for Rh<sub>0</sub>1h'. Similarly Rh<sub>2</sub> is short for Rh<sub>0</sub> and Rh<sub>1</sub>Rh is short for Rh<sub>0</sub>Rh<sub>1</sub>.

Factor Rh<sub>0</sub> is written with a capital letter to indicate that it is the most antigenic and therefore the most important clinically, and also that it has a special genetic position. Factors 1h' and 1h'' are written with small letters to conform with their lesser clinical importance and also to indicate that these two Rh factors are on an equal plain genetically and serologically, very similar to the agglutinogens A and B. Thus, considering factors 1h' and 1h'' alone four types of blood are possible which are analogous to the four Landstemeier blood groups. When factor Rh<sub>0</sub> is taken into account a double scheme of four types each results, as shown in Table II. Thus any one who understands the four blood groups immediately masters the eight Rh blood types.

To account for the heredity of the original Rh<sub>0</sub> factor, Landstemeier and Wiener<sup>3</sup> postulated the existence of a pair of allelic genes, *Rh* and *rh*. With the discovery of the eight Rh types, Wiener<sup>18, 19</sup> found it necessary to postulate

TABLE III EIGHT Rh TYPES AND THEIR TWENTY ONE GENOTYPES

Rh BLOOD TYPES	APPROXIMATE FREQUENCY IN A.A.C. (%)	POSSIBLE GENOTYPES
rh	13.0	rr
Rh	1.0	rr and rr
Rh	0.0	r r' and r' r
Rh Rh'	0.01	r r'
Rh	2.5	P <sup>0</sup> P <sup>0</sup> and P r
Rh	52.0	P <sup>1</sup> R <sup>1</sup> P <sup>1</sup> r R <sup>1</sup> r P <sup>1</sup> P and r P <sup>0</sup>
Rh	10.0	I <sup>2</sup> P R <sup>2</sup> r R <sup>2</sup> r P P <sup>0</sup> and r P <sup>0</sup>
Rh Rh	14.0	I <sup>1</sup> P <sup>2</sup> P <sup>1</sup> r and r' P <sup>2</sup>

the existence of a minimum of six allelic genes designated as  $H^0$   $R^1$   $R^2$   $r$   $r'$  and  $r''$  respectively. It will be noted that under this system the notations for genes and genotypes are different from the notations for the agglutinogens (or factors) and phenotypes so that ambiguity is avoided. In Table III are shown the genotypes corresponding to the eight phenotypes according to Wiener's theory of multiple allelic genes. The accuracy of this theory has been established by studies on a large series of families as well as by statistical studies on the distribution of the Rh blood types.<sup>10, 29\*</sup> For example in Table IV is given a summary of the author's studies<sup>29</sup> carried out on a total of 525 families with 948 children. In these studies only two seeming exceptions to the theory were encountered and both of these were proved to be due to illegitimacy.

TABLE IV SUMMARY OF AUTHORS FAMILY STUDIES TO DATE

MATING	NUMBER OF FAMILIES	NUMBER OF CHILDREN OF TYPES							TOTAL
		rh	Rh	Rh	Rh Rh	Rh	rh	rh	
rh x rh	12	25	0	0	0	0	0	0	25
rh x Rh	169	51	221	0	0	16	0	0	290
Rh x Rh	44	5	0	52	0	1	0	0	8
Rh x Rh Rh	61	(1)	5	1	0	0	0	0	107
Rh x Rh	7	3	0	0	0	9	0	0	12
Rh x rh	1	1	0	(1)	0	0	2	0	4
Rh x rh	2	2	0	0	0	0	0	5	7
Rh x Rh	73	10	124	0	0	5	1	0	140
Rh <sub>1</sub> x Rh <sub>2</sub>	37	11	25	11	19	1	3	0	70
Rh x Rh Rh	48	0	60	12	37	0	0	0	101
Rh x Rh	12	5	10	0	0	3	0	0	18
Rh x rh	8	1	7	0	0	0	2	0	10
Rh <sub>1</sub> x rh	6	2	3	0	3	0	0	0	8
Rh <sub>2</sub> x Rh	3	1	0	0	0	0	0	0	6
Rh <sub>2</sub> x Rh Rh	14	0	6	14	8	0	0	0	38
Rh x Rh	2	0	0	1	0	4	0	0	7
Rh <sub>2</sub> x rh	4	1	0	2	2	0	2	0	7
Rh Rh x Rh Rh	9	0	4	2	0	0	0	0	16
Rh Rh <sub>2</sub> x Rh	1	0	0	1	0	0	0	0	2
Rh Rh x rh	2	0	1	1	2	0	0	0	4
Rh Rh x Rh	2	0	1	0	1	0	0	0	2
Rh x Rh	1	0	0	0	0	1	0	0	1
Rh x rh	1	0	0	0	0	1	1	0	2
Total	525	139	519	153	68	41	13	0	948

\* The alternative between multiple alleles and completely linked genes was discussed in the time also by the writer and others in relation to the hercity of the ABO group (Wiener & S. Blood Groups and Transfusion, Springfield, Ill. 1947 Charles C. Thomas, Inc.) and (194). The English school have now received this discussion in relation to the Rh blood types.

Levine<sup>30</sup> and Javert<sup>31</sup> demonstrated that Rh-negative blood specimens contain a special agglutinin designated H<sub>1</sub> by them because of its apparently reciprocal relation to Rh. Race and Taylor<sup>32</sup> independently discovered an agglutinin which they called St, and showed that St was related to h' as M is related to N. Wiener and co-workers<sup>33</sup> have proved that H<sub>1</sub> and St are identical so that the designation St has been abandoned. Since the H<sub>1</sub> antigen first found was shown by Levine and Fisher to be reciprocally related to h', Wiener<sup>34</sup> suggested that it be designated as h<sub>1</sub>'. As Fisher<sup>35</sup> pointed out, theoretically three H<sub>1</sub> factors are possible, corresponding to the three Rh factors. Actually, h<sub>1</sub>' has been found<sup>36</sup> but convincing evidence regarding the existence of H<sub>10</sub> has not yet been published.<sup>†</sup>

Sensitization to the H<sub>1</sub> factors can cause clinical complications similar to those produced by Rh sensitization, namely intragroup hemolytic transfusion reactions and erythroblastosis fetalis. However, the H<sub>1</sub> factors are far less antigenic than the Rh factors, so that such cases are quite rare. For the same reason, H<sub>1</sub> antisera are much more difficult to procure than Rh antisera, and to date attempts to immunize H<sub>1</sub>-negative donors in order to produce such antisera have met with little or no success. Yet H<sub>1</sub> antisera are necessary to resolve clinical problems caused by sensitization to factors other than Rh, and for the selection of H<sub>1</sub>-negative donors to be used for transfusing patients sensitized to

TABLE V THE Rh H<sub>1</sub> BLOOD TYPES AND THEIR CORRESPONDING GENOTYPES

Rh BLOOD TYPES†	REACTION WITH SERUM		Rh SUBTYPES†	APPROXIMATE FREQUENCY IN NYC (%)	POSSIBLE GENOTYPES
	ANTI h <sub>1</sub> '	ANTI h <sub>1</sub> ''			
1h	+	+	1h	13.0	11
1h' }	-	+	1h'rh'	01	1'1'
	+	-	1h'1h	1.0	1'r
1h'' }	-	-	1h''1h''	0.05	1''1''
	-	+	rh''rh	0.5	1''1
1h'rh''	+	+	rh'rh''	01	1'r1''
Rh <sub>0</sub>	+	+	Rh <sub>0</sub>	2.0	R <sup>0</sup> R <sup>0</sup> and R <sup>0</sup> r
Rh <sub>1</sub> }	-	-	Rh <sub>1</sub> Rh <sub>1</sub>	20.0	R <sup>1</sup> R <sup>1</sup> and R <sup>1</sup> r
	+	+	Rh <sub>1</sub> rh	34.0	R <sup>1</sup> r, R <sup>1</sup> R <sup>0</sup> , and h <sub>1</sub> '
Rh }	+	-	Rh Rh	3.0	R-R- and R''
	-	+	Rh rh	12.0	R'r, R-R <sup>0</sup> , and R <sup>0</sup> r''
Rh <sub>1</sub> Rh	+	+	Rh <sub>1</sub> Rh	14.5	R <sup>1</sup> R <sup>0</sup> , h <sub>1</sub> 1'', and r <sup>1</sup>

\*Tests which need not be made because the reactions in these combinations are invariably positive except for types 1h'1h'' and Rh<sub>1</sub>Rh<sub>1</sub> in the case of individuals carrying the alleles R<sup>1</sup> and R<sup>2</sup>. Should these tests be done and negative reactions obtained there has been an error in technique and the entire examination should be repeated.

†These are phenotypes and the symbols have been selected so as to indicate what tests have actually been done and what reactions were obtained. For example type Rh<sub>1</sub> indicates that tests have been made only with sera anti-1h' anti-rh'' and anti Rh<sub>0</sub> and positive reactions obtained with anti-rh' and anti-Rh<sub>0</sub> and a negative reaction with anti 1h'' on the other hand type Rh<sub>1</sub>Rh<sub>1</sub> indicates type Rh<sub>1</sub> blood which also has been tested with anti h<sub>1</sub>' serum with which it gives a negative reaction.

\*A second example of anti-h<sub>1</sub>' has recently been found by Wiener and Peter.<sup>37</sup> The theoretical reactions of anti-H<sub>10</sub> sera have been presented by Fisher<sup>38</sup> and also by the writer<sup>34</sup> in previous papers. They are not included in the present paper because the writer has never encountered an anti-H<sub>10</sub> serum though such sera are said to have been found by others (Haberman S. Hill J. M. Everist B. W. and Davenport J. W. Jr. Blood in press).

one of the  $H_1$  factors. Another important application of the Hr antisera is for a presumptive test for heterozygosity or homozygosity of Rh positive husbands of Rh negative prospective mothers who are known to be sensitized to the Rh factor. Still another application is in disputed paternity cases since the Rh  $H_1$  tests considerably increase the chances of excluding a falsely accused man.

After a blood sample has been classified into one of the eight Rh blood types with the aid of the three Rh antisera anti  $h'$ , anti  $rh$ , and anti  $Rh$ , if indicated the blood can be subtyped with the aid of anti  $H_1$  sera. The  $H_1$  tests are done after the Rh tests in order to avoid unnecessary  $H_1$  testing so as to conserve the rare and valuable  $H_1$  antisera. Thus, only blood of types  $Rh_1$  and  $rh$  need be tested with anti  $h'$  serum and similarly only blood of types  $Rh$  and  $rh$  need be tested with anti  $h''$  serum. Other combinations uniformly give positive reactions, as shown in Table V, barring blood from the rare individuals who carry genes  $r'$  and  $R$ .<sup>38, 40</sup>

The Rh  $H_1$  subtypes are designated as follows:

$Rh_1 h' -$  is called  $Rh_1 Rh_1$   
 $Rh_1 h' +$  is called  $Rh_1 rh$   
 $rh h' -$  is called  $rh rh$   
 $rh h' +$  is called  $rh rh$

Similarly

$Rh h_2 -$  is called  $Rh_2 Rh$   
 $Rh h_2 +$  is called  $Rh rh$   
 $rh h_2 -$  is called  $rh rh$   
 $rh h_2 +$  is called  $rh rh$

It must be emphasized that these designations refer to *phenotypes* not *genotypes*. Thus corresponding to phenotype  $Rh_1 Rh_1$ , two genotypes  $R'R'$  and  $R'r$  are theoretically possible while corresponding to phenotype  $Rh_1 rh$  three genotypes,  $R'r$ ,  $R'R^0$ , and  $r'R^0$ , are possible. Of these five genotypes  $R'R'$  and  $R'r$  are by far the most common and it will be seen that this is the basis for the selection of the phenotype names, namely to indicate the most likely genotype. It is in this sense that anti  $h'$  serum can be used to determine the *probable* genotype of type  $Rh_1$  individuals and anti  $h'$  serum to determine the *probable* genotype of type  $Rh_2$  individuals.

From the foregoing it should be evident as has been pointed out by Wiener that the  $H_1$  factors hold a serologic and genetic position in the scheme of the Rh-Hr blood types analogous to that of the O factor in the less complicated scheme of the Landsteiner ABO groups. Just as the O factor is by far the least antigenic among the APO factors, similarly the two  $H_1$  factors are far less antigenic than the three (or four\*) Rh factors. In this connection it may be mentioned that clinical sensitization to the O factor has occurred producing fetal erythroblastosis entirely comparable to cases of erythroblastosis caused by Hr sensitization.\*

\*The existence of a fourth Rh factor  $rh''$  was predicted by Wiener<sup>40</sup> and confirmed by Callender and Race<sup>41</sup> who designated it  $C''$ .

## MATERIALS AND METHODS

The cases forming the basis of this study were derived principally from the Blood Transfusion Division of the Jewish Hospital of Brooklyn, though a number of cases from other institutions were also included. Before every transfusion 10 c.c. of blood were obtained from the patient by venepuncture, 5 c.c. of which were mixed with dried oxalate powder, as in the Wintrobe Landsberg sedimentation test, and used for the grouping, Rh, and cross-match tests, while 5 c.c. were placed in a dry clean tube and kept in the refrigerator for future reference. If any patient had a chill or other signs of reaction during or after the transfusion a second sample of blood was obtained without delay. The group and Rh type of the patient's and donor's blood specimens were verified, and the depth of color of the patient's serum (plasma) was compared with that of the pretransfusion sample. All glassware, syringes, and needles for these studies were sterilized in the hot air oven in order to avoid hemolysis due to moisture, and care was taken also when separating the clots to avoid artefacts caused by trauma to the clot. If the findings verified the original grouping and Rh report, and if there was no significant change in the color of the patient's plasma, the reaction was usually interpreted as pyrogenic and no contraindication was ordinarily considered to exist to further transfusions.\* In cases where the posttransfusion plasma was definitely darker than the pretransfusion sample, further studies were carried out to detect the possible presence of intra-group incompatibilities of an unusual type not provided against by the routine pretransfusion tests.

For simplicity in analyzing the results, the presentation will be confined to a statistical analysis of the groups, M N types and Rh Hr types of patients having reactions to transfusion, together with some brief clinical data concerning the reactions, and the results of tests for irregular isoantibodies in the patients' sera.

## RESULTS

In Table VI are listed thirty-two cases of posttransfusion febrile reactions. In Cases 1 to 22 there was some clinical evidence of posttransfusion hemolysis while in Cases 23 to 32 there was no evidence of hemolysis.

It will be seen that the distribution of the A-B O blood groups and M N types among the patients did not differ significantly from that occurring in the general population. On the other hand, while the distribution of the Rh Hr among the control cases (Cases 23 to 32) did not seem unusual, the distribution among the patients who had hemolytic reactions deviated strikingly. One can not help but be impressed by the large proportion of H<sub>1</sub>-negative patients in the series—fourteen Rh<sub>1</sub>Rh<sub>1</sub> and three Rh<sub>2</sub>Rh<sub>2</sub> out of twenty-two cases. These results suggest that sensitization to the H<sub>1</sub> factors, h<sub>1</sub>' and h<sub>1</sub>", must have played a role in many of the reactions.

Attempts to demonstrate the presence of irregular H<sub>1</sub> isoantibodies in the sera of the Hr-negative patients failed in all but three of the cases in this series. In a fourth case (Case 14) which has been reported elsewhere,<sup>14</sup> no H<sub>1</sub> antibody was found but instead a different irregular isoantibody reacting with about 14 per cent of all Caucasian blood and not corresponding to any hitherto described antibody†. Of the three cases showing the presence of H<sub>1</sub> isoantibodies, in two (Cases 21 and 22) the specificity corresponded to h<sub>1</sub>', while in the third (Case

\*If any of the blood used for transfusion was left in the transfusion bottle smear and cultures were made for the possible presence of molds or bacteria. In only one case contamination proved to be the cause of reactions in our series.

†A sample of this serum was sent to Dr. A. E. Mourant who stated that it gave reactions corresponding to his own so-called Kell serum. Since in the original report<sup>14</sup> on Kell antibody the serum was said to give only 7 per cent reactions instead of 14 per cent as obtained by us with anti-Si serum more work must be done before the two antigens may be assumed to be identical.



TABLE VI LIST OF CASES WITH FEBRILE REACTIONS TO BLOOD TRANSFUSIONS

CASE	PATIENT'S BLOOD TYPE	OTHER FINDINGS
1	A NRh Rh	Rise in icterus index
2	A MRh Rh <sub>1</sub>	Rise in icterus index *
3	A Rh Rh	Rise in icterus index *
4	OMRh Rh	Rise in icterus index *
5	BMNRh Rh	Rise in icterus index *
6	A MNRh Rh	Icterus index rose from 5 to 12 units
7	OMRh Rh <sub>2</sub>	Rise in icterus index all defined irregular antibody detected in serum at times
8	A MNRh rh	Icterus index rose from 1 to 8 units
9	A MNRh Rh	Icterus index rose from 10 to 12 unit
10	A MNRh Rh <sub>2</sub>	Hemoglobinuria
11	ONRh Rh <sub>1</sub>	Cyanosis drop in hemoglobin
12	ONRh Rh	Icterus index rose from 2 to 4 units
13	OMNRh rh	Icterus index rose from 2 to 4 unit
14	AMNRh <sub>1</sub> Rh	Shock drop in hemoglobin concentration irregular antibody anti S <sub>1</sub> demonstrable by coagulation technique
15	A NRh Rh <sub>1</sub>	Rise in icterus index from 2 to 3 unit
16	OMRh Rh	Rise in icterus index
17	BMNRh	Rise in icterus index
18	OMRh Rh <sub>2</sub>	Strong anti hr agglutinin demonstrable in patient serum
19	A MRh Rh	Collapse severe hemoglobin urea
20	OMNRh Rh	Rise in icterus index from 4 to 12 unit dark urine
21	A BMNRh Rh	Strong anti hr agglutinin demonstrable in patient serum
22	BMNRh Rh	Patient's blood showed evanescent rise in hemoglobin concentration then a progressive fall terminating with patient death anti hr agglutinins in patient serum
23	ONRh Rh	No change in icterus index †
24	AMNRh <sub>1</sub> rh	No change in icterus index †
25	ORh rh	No change in icterus index †
26	OMNRh rh	No change in icterus index †
27	ONRh Rh	No change in icterus index †
28	A MNRh Rh <sub>2</sub>	No change in icterus index †
29	A NRh rh	No change in icterus index †
30	A NRh rh	No change in icterus index †
31	OMRh Rh	No other data
32	OMRh rh	No other data

The posttransfusion plasma was darker to the eye than the pretransfusion plasma but no actual measurement of the icterus index was made

†The depth of color of the pre and posttransfusion plasmas appeared the same to the eye

18) the specificity proved to be hr'. This case of hr' sensitization was the first with demonstrable antibodies of this specificity to be encountered since the original report of Mourant<sup>29</sup> and therefore has been reported in detail elsewhere.<sup>3</sup> With regard to the two cases of hr' sensitization with demonstrable antibodies the scanty clinical data available concerning these patients are presented below for the first time

CASE 1.—This patient was brought to our attention by Dr William Thillmer who had encountered difficulty in finding compatible blood for transfusing the patient because of the presence of an irregular isogglutinin. The patient was being treated for a spinal cord tumor.

The patient had had two normal children, the 20 year old son and 16 year old daughter were both well. She never had had any miscarriages or stillbirths.

On Feb 17, 1939, the first exploratory operation had been performed. According to the patient he received her first blood transfusion in 1945. During that year the patient received three transfusions which were followed by chill. (The patient was not sure whether all the transfusions were followed by reactions.)

During the hospital admission of this study Nov 19 1947, the patient was given a transfusion of 500 cc of group AB Rh positive blood following which he had a mild chill.

At the Montefiore Hospital Bronx, N. Y. we are indebted to Dr H. M. Zimmerman for his cooperation in obtaining additional blood for study.

A second transfusion, this time of 300 cc of group A, Rh negative blood, was given Nov 14, 1947, and this was followed by a severe chill. A blood count done on Nov 14, 1947, showed a hemoglobin concentration of 9.5 Gm per 100 cc, RBC, 3.30 million per cubic millimeter, WBC, 14,100 per cubic millimeter, polymorphonuclears, 76 (six band forms), lymphocytes, 23, and monocytes, 1. A repeat blood count of Dec 10, 1947, showed a hemoglobin concentration of 10 Gm per 100 cc, RBC, 3.70 million, WBC, 7,600, polymorphonuclears, 4 (four band forms), lymphocytes, 25, monocytes, 5.

Our own studies on the patient's blood showed her to belong to group AB, subgroup A<sub>1</sub>B, type MN, and type Rh<sub>1</sub>Rh<sub>1</sub>. Since the patient was H<sub>1</sub> negative (or more exactly, H<sub>1</sub>' negative), this suggested that the irregular antibody in the serum might be an anti-rh' agglutinin. In fact, titrations of the patient's serum at body temperature proved that powerful anti-rh' agglutinin was present, the titer of the serum for homozygous hr' positive blood (for example type rh) being approximately 250 units, while the titer for heterozygous hr' positive blood (for example type Rh<sub>1</sub>rh) was about half as high. However the serum exhibited a tendency to clump most if not all blood specimens of type Rh<sub>1</sub>Rh<sub>1</sub>, particularly when the mixtures were allowed to cool. Titrations at refrigerator temperature showed that this was due to the presence of a strong autoagglutinin. This interfering antibody could be removed by repeated absorptions with type Rh<sub>1</sub>Rh<sub>1</sub> of any blood group and in that way it was possible to obtain a satisfactory anti-rh' reagent for subtyping Rh<sub>1</sub> and type rh' individuals. Some of the tests indicated the possible presence of still a third abnormal antibody in the patient's serum, and, while this antibody was not studied carefully, in view of the patient's subgroup (A<sub>1</sub>B) the likelihood is that its specificity was anti O.

CASE 22—An intern at a hospital in New York City consulted us concerning the following transfusion problem.

The patient, a woman, was said to have pernicious anemia with cardiac complications. The pregnancy history was not known, but the patient was said to have been transfused several times approximately a year previously. On admission to the hospital on Nov 7, 1947, the patient's condition was critical, the blood having a hemoglobin concentration of only 2 Gm per cent, with RBC 1.1 million per cubic millimeter and 0.4 per cent reticulocytes on the smear. The patient was given an immediate transfusion of a total of 1,500 cc of blood and placed on liver therapy. A blood count of November 10 showed that the hemoglobin concentration had risen to 8 Gm per cent, with RBC 2.4 million and 0.4 per cent reticulocytes on the smear. By November 12 the hemoglobin concentration had fallen to 7 Gm per cent with RBC 1.48 million, though there were now 4.8 per cent reticulocytes on the smear, and this time the icterus index was found to be 32 units. By the next day the hemoglobin concentration had dropped to only 4 Gm per cent with a red blood cell count of 1.0 million while the icterus index rose to 75 units. The patient was then given another transfusion of 500 cc of blood, this time without any reaction. The following day the patient was chills and blood was referred to us for study.

In the blood typing tests the following reactions were obtained.

REACTIONS WITH ANTISERA OF SPECIFICITY							
A	B	M	N	rh'	rh''	Rh <sub>0</sub>	hr
-	++±	++±	$\frac{1}{10}(++)$	++	$\frac{1}{10}(+)$	++±	$\frac{1}{10}(++)$

These results indicated that the patient belonged to group B, type M, type Rh<sub>1</sub>Rh<sub>1</sub>, but that her body also contained some group B, type MN, type Rh<sub>1</sub>Rh<sub>1</sub> blood, probably remaining from the transfusion received the day before. This led us to entertain the likelihood that a developing sensitization to the hr' factor was the cause of the rapid elimination from the patient's body of the 1,500 cc of blood she had received the week previous. Examination of the patient's serum did reveal the presence of an irregular agglutinin.

most active at body temperature, which did not clump her own blood cells or blood from other type Rh,Rh<sub>1</sub> individuals of groups B and O confirming the prediction that she was sensitized to the hr' factor. The hr' agglutinin was weak however so that while blood specimens homozygous for the hr' factor, for example, types rh and Rh were clumped up to dilution 1:4, blood heterozygous for the hr' factor for example types Rh<sub>1</sub>rh and Rh Rh<sub>1</sub> was not clumped or only feebly clumped. The low titer of the hr antibody explained the continued presence in the patient's circulation of some of the type Rh<sub>1</sub>Rh blood received by transfusion the day before.

In view of these findings it was advised that for further transfusions to this patient only hr negative (type Rh Rh<sub>1</sub>) group B blood be used. Unfortunately the patient died before another transfusion could be given.

The cases listed in Table VI, and especially the two cases just described demonstrate that H<sub>1</sub> sensitization may pose a serious problem where patients require repeated transfusions over a long period of time. As has been demonstrated elsewhere,<sup>48-50</sup> the wide spacing of the transfusions is probably the most important factor in the development of isosensitization. For example two or more transfusions spaced widely apart as in the cases just described are much more likely to induce sensitization than a large series of transfusions given within a short period of time. In fact this principle has been applied successfully in the production of anti Rh sera of specificities Rh<sub>0</sub>, rh' and rh'' though we have not yet succeeded in producing anti H<sub>1</sub> sera in this manner. Two of our type rh donors,<sup>48</sup> both of group AB type N formed agglutinins of specificity anti M as well as antibodies of specificities Rh<sub>0</sub> and rh'' when injected with OMRh<sub>1</sub>Rh blood.<sup>50</sup> These observations lead one to draw certain analogies between the three systems of blood group and type antigens as shown in Table VII.

TABLE VII ANALOGIES AMONG ABO MN AND Rh Hr SYSTEM OF ANTIGENS

SYSTEM	MAJOR AGGLUTINOGENS (STRONGLY ANTIGENIC)	MINOR AGGLUTINOGENS (WEAKLY ANTIGENIC)
A B O	A B C + A	O (A) †
M N	M	N
Rh Hr	Rh <sub>0</sub> rh rh	hr' hr ‡

For simplicity the rarer types A<sub>2</sub> M<sub>2</sub> N and so on are not included in this table.

†The common antigen shared by blood containing agglutinin A or B or both.

‡The antigen shared by agglutinogens O and A but absent from agglutinogens A and B.

§Convincing evidence concerning the existence of the theoretically possible antigen Hr has not yet been published.

Recently we were consulted concerning a case which offered an unusual problem of interest in connection with the subject under discussion. The patient was a boy requiring repeated transfusions over a period of years, and we were asked for advice in the selection of donors in order to avoid if possible the development of isosensitization. Grouping and Rh Hr tests on this patient and the parents gave the results shown in Table VIII. As can be seen this patient offered a problem practically impossible to solve. Since he belonged to type rh<sub>1</sub>, his blood lacked the Rh<sub>0</sub> agglutinin and so he had to be given Rh negative blood. At the same time, he was hr' negative and it was desirable also to avoid the possibility of H<sub>1</sub> sensitization. Unfortunately the only type of blood which is both Rh<sub>0</sub> negative and hr' negative is blood of the rare type rh'rh' identical

TABLE VIII

BLOOD OF	GROUP	M N TYPE	Rh Hr TYPE	
			PHENOTYPE	GENOTYPE
Father of patient	A <sub>1</sub>	N	Rh <sub>1</sub> Rh <sub>2</sub>	rR <sup>r</sup>
Mother of patient	A <sub>1</sub>	MIN	Rh <sub>1</sub> Rh <sub>1</sub>	Rir <sup>r</sup>
Patient	A <sub>2</sub>	N	rh'r <sup>r</sup>	r <sup>r</sup>

with the patient's blood and with an incidence of only 1 in about 10,000 in individuals. In addition, the patient belonged to type N, which has a frequency of 20 per cent, so that a completely compatible donor, even using group O donor, would occur only once among about 50,000 individuals. In this case, therefore, we had to be content to take into account only the most antigenic of the three factors Rh<sub>0</sub>, hr', and M, and so ordinary type 1h donors of group A were used. Incidentally, the case offers an interesting example of the genetics of the Rh Hr types as shown in Table VIII.

## COMMENT

Our attention was first called to the possibility of hemolytic transfusion reactions caused by isosensitization to the H<sub>1</sub> factor by a patient seen in 1944. In this case, reported by Speck and Sonn,<sup>44</sup> the patient, who belonged to type Rh<sub>1</sub>Rh<sub>1</sub> and group AB, had a hemolytic reaction during a post partum transfusion of type 1h, group AB blood. However, as in most of the cases described in this paper, abnormal isoantibodies were not clearly demonstrable in the patient's serum. More recently Sussman<sup>45</sup> has reported a case of hr' sensitization by transfusion in which the patient's serum contained strong isoantibodies which, however, were demonstrable only by the conglutination technique.

In view of our findings it is necessary for all hospitals giving large numbers of transfusions, especially to patients requiring repeated transfusions spread over a long period of time, to have available not only type 1h but also type Rh<sub>1</sub>Rh<sub>1</sub> and type Rh<sub>2</sub>Rh<sub>2</sub> donors, at least of group O, for use for isosensitized patients. For the present it is still not practicable to carry out H<sub>1</sub> tests on all patients requiring transfusions for selecting donors. Since the Hr factors are only poorly antigenic, we ordinarily disregard them in the pretransfusion tests. Only when a patient has had a reaction do we test for the H<sub>1</sub> factors, and if such a patient is found to belong to type Rh<sub>1</sub>Rh<sub>1</sub> or type Rh<sub>2</sub>Rh<sub>2</sub>, the precaution is taken to use only Hr-negative donors for future transfusions. Ideally, however, the tests should eventually become part of the routine pretransfusion selection of donors, not only to prevent reactions but also to prevent sensitization, especially of women who might subsequently have children with erythroblastosis caused by H<sub>1</sub> sensitization.<sup>46</sup>

## SUMMARY

The introduction of routine Rh testing along with blood grouping tests on a basis of the selection of donors for transfusions has served to eliminate the danger of serious hemolytic reactions. Simultaneously there has been a reduction in the frequency of posttransfusion chills, as a result of the perfection of methods of eliminating pyrogenic materials from blood transfusion apparatus. Thus at the author's institution the frequency of febrile reactions dropped in

79 per cent in 1936 to 29 per cent in 1939 and to only 12 per cent by 1947. The virtual elimination of pyrogenic reactions has served to make more prominent another class of hemolytic reactions—usually only of minor severity, occurring in Rh positive patients becoming sensitized by repeated transfusions given over a long period of time.

In a series of twenty three Rh positive patients having febrile reactions and at the same time showing evidence of posttransfusion hemolysis as many as seventeen were  $H_1$  negative (fourteen  $Rh_1Rh_1$  and three type  $Rh Rh$ ) while among ten patients with febrile reactions but without evidence of hemolysis none were  $H_r$  negative. This indicates that  $H_1$  sensitization plays a predominant role as a cause of hemolytic reactions in Rh positive patients.  $H_1$  antibodies were clearly demonstrable in the sera of only three (two anti  $hr'$  and one anti  $hr'$ ) of the seventeen presumably sensitized patients. This indicates that  $H_1$  sensitization, when it occurs at all, is usually mild in degree. This conforms with the usual mild course of reactions caused by  $H_1$  sensitization in that such reactions are usually so harmless that they are passed off as ordinary pyrogenic reactions. However, that such reactions may sometimes endanger the life of the patient is demonstrated by the fact that one of the three patients with demonstrable antibodies died.

While routine pretransfusion  $H_1$  typing is still not practicable one should at least investigate every febrile reaction for evidence of hemolysis. If hemolysis has occurred even though the patient is Rh positive  $H_r$  tests should be done and if the patient is found to be  $H_r$  negative only  $H_1$  negative blood of a compatible blood group should be used for future transfusions. If Rh negative patients have reactions despite transfusions of type rh blood, one should search for other sensitizations particularly against the M factor. Particularly difficult to solve will be instances of multiple sensitization one of the most common examples of which in the author's experience is double sensitization to factors Rh and M.

Eventually blood transfusion practice should include suitable precautions to avoid sensitization against the  $H_r$  factors as well as the Rh factors particularly in the case of women in order to avoid the birth of babies with erythroblastosis caused by  $H_1$  sensitization.

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# INFECTIOUS HEPATITIS INADVERTENTLY TRANSMITTED WITH THERAPEUTIC MALARIA

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**A**CU TE hepatitis resulting from the transmission of an ieterogenic agent in blood and blood products has been described frequently in recent years. The purpose of this paper is to call attention to a particular mode of transmission. It has been recognized that an ieterogenic agent might possibly be transferred when patients are inoculated with therapeutic malaria. However, the hazard has not received sufficient emphasis to cause suitable precautions to be taken in many hospitals using this type of fever therapy. The menace is particularly great since the usual efforts to perpetuate a strain of malaria also mean serial transmission of the ieterogenic agent.

The ieterogenic agent has been shown to be a virus.<sup>2</sup> Havens<sup>10</sup> has presented evidence that the ieterogenic agents of infectious hepatitis and homologous serum jaundice are different viruses. He bases his argument primarily on the fact that the incubation period of the former is 15 to 34 days and of the latter, 58 to 134 days. In 1943 Beeson<sup>1</sup> reported seven cases of jaundice occurring one to four months following transfusions. Other authors<sup>5</sup> have reported a mean incubation period in homologous serum jaundice of 100.1 days. According to the criteria of Havens, the cases to be presented in this paper would be classified as infectious hepatitis rather than homologous serum jaundice. The most common sources of infection have been contaminated lots of yellow fever vaccine,<sup>2, 4</sup> pooled plasma,<sup>6, 8</sup> and mumps convalescent plasma.<sup>3</sup> In 1947 Chalmers<sup>9</sup> reported on 490 patients who had been given therapeutic malaria. Of this group, thirty-six cases developed jaundice and evidence of liver damage. He felt that his cases were suggestive of the transfer of an ieterogenic agent at the time of inoculation with malaria. In Chalmers' series ninety-nine patients were mosquito inoculated and none of them developed jaundice.

We shall describe in this paper six patients who received serial inoculations of the same strain of quartan malaria and subsequently developed severe jaundice and marked evidence of hepatocellular damage. These cases were discovered when one of the convalescing patients was received by one of the authors in an intrahospital transfer, consequently, full study of these patients was undertaken only after they had begun the clinical recovery phase. For this reason all the laboratory data that might be desired were not obtained during the acute phase of the illness. The patients had been given malarial fever therapy in treatment of central nervous system syphilis. They were all men whose ages ranged from 29 to 51 years, and all were considered good risks for fever therapy with malaria.

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Prior to inoculation there was no clinical evidence of liver disease, and no hepatoxic drugs were given during hospitalization. The incubation period in this series ranged from 20 to 43 days, with an average of 32.5 days. The estimated incubation period in some instances is probably longer than the actual period since the patients rarely expressed any subjective symptoms. The sequence of, and the time interval between, the inoculations will be stressed in the case reports. The results of the bromsulfalein tests were obtained by using the 5 mg. per kilogram dose. The standard technique for inoculation with malaria at this hospital has been to give 5 cc. of donor blood intravenously and 5 cc. intramuscularly.

#### REPORT OF CASES

**CASE 1**—This patient (P. W.) was probably the original source of the virus in this series. All persons who had received this strain of malaria previously showed no evidence of infectious hepatitis. The six subsequent patients inoculated did develop marked evidence of infectious hepatitis. In later questioning P. W. admitted that two years earlier in the South Pacific several of his tent mates had developed jaundice and that he had had an episode of malaise with vague abdominal distress. He had had no further symptoms until the present illness. The patient was a 34-year-old white man who was inoculated with malaria May 5, 1947. His fever was terminated June 29, 1947, after fifty-two hours above 104°. At no time was jaundice noted nor were symptoms of hepatitis present. During the course of fever the red blood cell count fell from 5,210,000 to 4,100,000 per cubic millimeter. The cephalin flocculation test 139 days after inoculation was positive plus 4 after twenty-four hours and on the same date the bromsulfalein test showed 9.5 per cent retention after thirty minutes. The patient has continued free of any hepatic symptoms.

**CASE 2**—J. I., a 41-year-old white man, was inoculated May 16, 1947, with blood from P. W. (Case 1). The patient complained of malaise and anorexia forty-one days after inoculation, and on July 5, 1947, jaundice and symptoms of hepatitis had become so severe that the malaria was terminated. During the course of fever the red blood cell count fell from 5,400,000 to 3,230,000. A leucopenia did not develop. On the forty-eighth day following inoculation the icterus index was 100 units and serum protein was 8.25 Gm. per 100 cc. with albumin 3.45 Gm. per 100 cc. and globulin 4.8 Gm. per 100 cubic centimeters. The temperature curve did not fall to the base line between the spikes of fever and after the malaria was terminated a low grade temperature elevation continued for twenty days.

**CASE 3**—F. S., a 40-year-old white man, was inoculated with malaria on July 3, 1947, with blood from J. I. (Case 2). He complained of nausea and vomiting seventeen days after inoculation, and two days later marked jaundice appeared. Abdominal pain was present and there was tenderness at the right costal margin though the liver could not be palpated. His fever was terminated twenty-six days after inoculation. The red blood cell count did not fall during the fever therapy possibly because of dehydration. Between spikes of fever due to malaria the temperature curve did not return to the base line. The icterus index twenty-one days after inoculation was 90 units, at seventy-seven days 159 units and at 109 days 13 units. The cephalin flocculation test seventy-seven days after inoculation was positive plus 4 at the end of twenty-four hours and thirty days later had become negative. The bromsulfalein test eighty-five days after inoculation showed 37 per cent retention at the end of thirty minutes.

**CASE 4**—W. B., a 51-year-old white man, was inoculated with malaria on July 29, 1947, with blood from F. S. (Case 3). Jaundice appeared thirty-eight days after inoculation and termination of fever was begun. The patient had twenty-six hours of fever above 104°. The initial red blood cell count of 4,880,000 and hemoglobin of 13.2 Gm. fell during fever to 3,900,000 and 9.2 Gm. respectively. The white blood cell count was never above 7,000 nor

below 5,400 The bromsulfalein test ninety four days after inoculation showed thirty per cent retention at the end of thirty minutes Seventy five days after inoculation the icterus index was 9.6 units and the cephalin flocculation was positive plus 4 after twenty four hours At 111 days after inoculation cephalin flocculation was still positive plus four after twenty four hours

CASE 5—C H, a 39 year old white man, was inoculated with malaria on July 29, 1947, with blood taken from F S (Case 3) Jaundice appeared forty three days after inoculation and was accompanied by severe abdominal pain, eructation, nausea, and vomiting These symptoms were so severe that termination of malaria was necessary forty eight days after inoculation During this illness the red blood cell count fell from 4,550,000 to 3,330,000, and the hemoglobin from 13.2 to 10.6 grams A leucopenia did not develop in this patient Bed rest was required for three weeks after malaria was terminated The bromsulfalein test showed 25 per cent retention at thirty minutes seventy three days after inoculation Seventy five days after inoculation the icterus index was still 16.7 units and cephalin flocculation was positive plus 4 after twenty four hours

CASE 6—R T, a 34 year old white man, was inoculated with malaria from F S (Case 3) on July 29, 1947 Jaundice appeared twenty seven days after inoculation, and the patient appeared acutely ill with sustained fever and gastrointestinal distress The fever did not fall below 101° between the malaria spikes Malaria was terminated thirty days after inoculation At the peak of the acute illness, the previously normal red blood cell count and hemoglobin fell to 3,590,000 and 13.2 Gm respectively The white blood cell count fell to 3,800 Sixty three days after inoculation the cephalin flocculation was positive plus 4 at the end of twenty four hours Sixty six days after inoculation the bromsulfalein test showed 29.9 per cent retention at the end of thirty minutes, and cephalin flocculation was positive plus 2 at the end of forty eight hours Cephalin flocculation was negative after forty eight hours 111 days after inoculation

CASE 7—W S, a 43 year old white man, was inoculated with malaria from C H (Case 5) on Sept 5, 1947 Jaundice appeared twenty seven days after inoculation and the patient complained of abdominal pain, anorexia, nausea, and vomiting The fever curve rarely fell below 100° between the malaria spikes A tender liver edge was palpated 3 cm below the right costal margin Termination of malaria was necessary thirty one days after inoculation The patient had a long convalescence with continuation of gastrointestinal symptoms Before inoculation with malaria the red blood cell count was 5,210,000 and the hemoglobin was 10.4 grams During the acute phase of illness the red blood cell count fell to 3,280,000 and the hemoglobin to 10.6 grams The white blood cell count remained normal A low grade fever continued for three weeks after termination of malaria Thirty five days after inoculation there was 39 per cent bromsulfalein retention after thirty minutes Thirty eight days after inoculation the icterus index was still 14.3 units The cephalin flocculation test was positive plus 2 at forty eight hours, seventy one days after inoculation

#### COMMENT

The consecutive development in these patients of evidence of hepatitis which followed serial inoculation with malaria is significant The fact that all the findings could have been caused by a virulent strain of malaria is acknowledged. However, this same strain of malaria had been used on a great number of other individuals in this hospital without such ill effects It is felt that the only logical explanation is that an icterogenic agent was transferred at the time of inoculation with malaria According to Havens' classification, the short incubation period in this series is more compatible with infectious hepatitis than with homologous serum jaundice The transfer of an icterogenic agent, therefore, constitutes an additional hazard in malarial fever therapy In addition to the

obvious danger to the patient this complication necessitates early termination of the fever. The patient who can be a source of the ieterogenic agent should not have his blood passed to the next patient, and will probably be a bad risk for fever therapy with malaria as well. It would seem that the best way to avoid this hazard is to perform liver function tests routinely before fever therapy is begun.

# SUMMARY

Six patients are presented who consecutively developed evidence of hepatitis following inoculation with therapeutic malaria. An additional patient is described who probably served as the original source of the ieterogenic agent in this series. It is believed that infectious hepatitis was inadvertently transmitted with therapeutic malaria.

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# STUDIES IN HODGKIN'S SYNDROME

## VII NITROGEN MUSTARD THERAPY

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THE occurrence of leucopenia in individuals exposed to nitrogen mustard gas was recognized during World War I.<sup>1</sup> It was not until World War II however that extensive studies concerning the cytotoxic properties of nitrogen mustard compounds were undertaken. A specific affinity of the *bis* and *tris* ( $\beta$  chloroethyl) amines for rapidly growing and regenerating tissue in general and a particular susceptibility of the hematopoietic and lymphoid systems to the cytotoxic action of these substances have been described.<sup>1, 2, 11</sup> The therapeutic trial of methyl-*bis* and *tris* nitrogen mustard compounds in lymphomatous and certain other neoplastic diseases followed. Gilman and Philips<sup>10</sup> and Rhoads<sup>4</sup> have summarized the historical background as well as the chemical, pharmacologic, and physiologic properties of this group of chemical agents.

Clinical studies using the *tris* and the *bis* ( $\beta$  chloroethyl) amines have been carried on by a number of investigators. Patients with a variety of neoplasms exclusive of the hematologic dyscrasias have been treated with these compounds but results in most cases do not appear to justify their continued use. The most encouraging results described to date have been observed in Hodgkin's disease.<sup>12-15</sup> It is generally agreed, on the basis of preliminary published data, that remissions which are induced by this therapy are temporary, lasting 0 to 8 months, and that the clinical results achieved are comparable in many ways to those produced by roentgen rays.<sup>5, 9, 12, 14, 15</sup> Kainofsky has restated an opinion shared by many investigators<sup>9</sup> that roentgen therapy is advisable in early localized Hodgkin's lesions and local extensions, and that nitrogen mustard may be more effective in generalized Hodgkin's disease, early and late and in cases characterized by severe systemic intoxication.

The only persistent systemic toxic effect of the halogenated amines reported at so-called therapeutic dose levels is a more or less severe but transient damage to hematopoietic and lymphoid tissues. Leucopenia, thrombocytopenia, and moderate anemia appear in a large percentage of cases under these conditions. A decrease in the number of formed elements in the peripheral blood becomes apparent three to four days after the beginning of therapy and continues for approximately three weeks. Serial bone marrow studies have revealed progressive destructive hypoplasia consistent with the peripheral blood findings. Dur

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ing the fourth week regeneration takes place<sup>6, 8, 1</sup>. The first cellular elements to be depressed are the lymphocytes followed in a few days by an absolute panleucopenia with a decrease in the number of polymorphonuclear leucocytes.

Jacobson and associates<sup>1</sup> have described a left shift in the lymphocytes with the appearance of abnormal forms. Similar qualitative abnormalities have been noted in the monocyte series. Both cell types return to normal within two or three weeks. The reticulocytes of the peripheral blood are reduced during the first week to less than 0.1 per cent and thrombocytopenia develops during the third week after treatment. However in spite of this obvious and extensive damage to the bone marrow the great majority of patients revert promptly to a relatively normal hematologic equilibrium within a few weeks after therapy depending on the degree of normality of the marrow before therapy was instituted. Folic acid, Pentnucleotide, iron and whole blood do not ameliorate the toxic effects produced.

The present report is based on the treatment of thirty one patients with Hodgkin's disease with forty four courses of the methyl bis ( $\beta$  chloroethyl) amine hydrochloride. In every case the biopsy diagnosis of Hodgkin's disease was confirmed by two or more pathologists before treatment was begun. Two patients received three, nine received two and twenty received one course of therapy. For the most part the choice of patients in this series followed the criteria of Karnofsky<sup>9</sup> in addition patients were selected who presented evidence of local extensions of the disease and who had not responded to roentgen therapy. Serial peripheral blood studies were made in all patients supplemented whenever practicable by sternal marrow examinations. The supravitral staining technique was used in all differential blood cell estimations.

Each patient was given 0.1 mg of HN<sub>2</sub> per kilogram body weight for five days with the exception of the seven individuals who were given a double dose on two consecutive days and a single dose on the third day. Each dose was dissolved in 10 cc of normal saline and injected immediately via the tube through which normal saline was being administered intravenously to the patient. From 200 to 500 cc of saline were infused during each injection of HN<sub>2</sub>.

In evaluating the results obtained after nitrogen mustard therapy a remission was defined as a decrease in unfavorable symptoms ascribed to the disease and an objective regression of the disease as measured by roentgenologic and hematologic findings and physical examination.

#### RESULTS

A summary of the results is presented in Table I. The average remission time following HN<sub>2</sub> in this series was 2.8 months. The longest remissions were four, five and six months respectively. Some of these cases are still in remission at the time of writing. Ten patients included in this series have expired. The remainder have been maintained on subsequent courses of nitrogen mustard and/or radiation therapy.

An analysis of the results of treatment was made with special reference to the therapy used previous to the initial course of mustard; the specific indica-

TABLE I

CASE AND PATIENT	COURSE OF THERAPY	DURATION OF ILLNESS	PREVIOUS X RAY THERAPY	NUMBER OF DOSES	LENGTH OF TIME OF REMISSION (MO)	SKIN ERUPTION	ANEMIA	PER CENT CHANGEL RETICULO CYTE	BONE MARROW		APPEAL ANCHOR OF BLOOD EFFECT (DAYS)	SUPPLEMENTAL THERAPY		EXPIRED
									HODG KIN'S IN VOLVE MENT	HYPO PLASIA AFTER TREAT APY		WHOLE BLOOD	FOLIC ACID	
1 P D	1	3 yr	+	5		+	0	2402	++	0	33	0	0	
	2			5		+	+	2410	+++	++	18	+	+	
2 V V	3			3		+	+++	5226	+	+++	25	+	0	+
3 A H	4	2 yr	+	5	2	+	+	2206	0	++	7	0	0	
4 N L	5	5 mo	+	5	1	+	+	1820	0	+++	23	0	+	+
	6	2 yr	+	5		+	+++	0268	0	++	40	0	0	+
5 O S	7			5		+	++		0	+++		+	0	
	8	2 yr	+	5		0	0		0	+		0	0	
	9			5	2	+	+		0	+		0	0	
6 E F	10			5	1	+	0		0	+		0	0	
7 C M	11	2 yr	+	5		+	0		0	+		0	0	
8 F N	12	6 yr	+	5	5	+	+	0824	0	++	29	0	0	
9 M G	13	4 yr	+	5	6	+	0		+	+++	18	0	0	
	14	1 yr	+	5	2	+	0	2200	+++	+++	25	0	0	
10 C L	15			5		+	+++		0	+	11	+	+	
11 D R	16	3 1/2 yr	+	5	4	0	0		+++	+++	18	0	0	
12 C L	17	3 yr	+	5	1	+	0	0202	++	+++	10	+	+	+
	18	2 1/2 yr	+	5	2	+	+++	2216	+++	+++	35	0	0	
13 B	19			5		+	+	0200	+++	+++	15	+	+	+
	20	2 yr	+	5		+	+		+++	+++	15	0	0	+



tions for  $\text{HN}_2$ , and the subsequent activity and treatment of the disease. The case numbers used refer to Table I. The patients who obtained benefit from nitrogen mustard therapy were divided into four groups. The first group includes one patient who had received no previous therapy of any kind (Case 29) and those patients who had previously experienced remissions following roentgen radiation (Cases 2, 12, 16, 23, 24, and 25). Improvement was observed in all members of this group after nitrogen mustard. The second group (Cases 5, 8, 10, 20 and 30) includes patients with widely disseminated Hodgkin's disease manifest by severe systemic intoxication but no demonstrable localized foci of activity. Following treatment with  $\text{HN}_2$  remissions occurred and later recurrences of the disease were localized and responded readily to roentgen ray therapy. The third group consists of patients (Cases 3, 7, 9, 11, 14, 18, 21, and 31) who had received radiation over localized areas of activity without any beneficial effect and were considered to be roentgen ray resistant. These patients demonstrated significant clinical improvement after  $\text{HN}_2$  therapy. The fourth group is represented by three patients (Cases 4, 6, and 19) who did not improve following roentgen therapy and who also failed to improve after nitrogen mustard. Subsequent radiation, however, produced remissions in these patients. In the first three groups mentioned clinical remissions were observed following a total of twenty-four courses (55 per cent) and in twenty patients (65 per cent). In summary, a total of twenty-three patients received benefit from nitrogen mustard therapy either through an immediate remission or, indirectly, by an apparent re-establishment of sensitivity to roentgen rays. Since three of the thirty-one patients in this series (Cases 13, 26, and 27) did not return for follow-up studies after discharge from the hospital, the twenty-three who obtained benefit following  $\text{HN}_2$  therapy represent 82 per cent of the twenty-eight cases in which subsequent clinical and laboratory observations could be made. Five patients (Cases 1, 15, 17, 21, and 28) were not benefited by  $\text{HN}_2$  and subsequently expired.

In those patients in whom a remission was observed, the most troublesome symptoms of active Hodgkin's disease such as pain, pruritis, and occasional anorexia were relieved frequently after the third or fourth injection of the series. Fever was the most consistent clinical finding during exacerbations of Hodgkin's disease. In those cases in which a remission occurred, the temperature returned to normal during therapy or within a week after its completion. Many patients who noted no sustained subjective or objective evidence of regression of the disease nevertheless described a moderate degree of temporary relief from one or more of their symptoms. One patient (Case 14) developed a marked diffuse brown pigmentation of the skin during one exacerbation of the disease. Nitrogen mustard therapy was given and two weeks after completion of therapy the pigmentation had disappeared with the reappearance of a normal skin color. This decrease in abnormal skin pigmentation also was observed to a lesser degree in two other patients (Cases 2 and 9).

The toxic effects of the methyl-bis compound are fairly consistent. With the exception of one case in which diarrhea was present, anorexia, nausea, and vomiting were the only immediate toxic reactions. These symptoms occurred



during thirty eight of the forty four courses given and were usually apparent within one half to three hours following the administration of therapy. In some instances nausea alone of short duration was described. In others nausea and vomiting of twenty four to forty eight hours duration were noted. Thrombophlebitis at the site of injection occurred during four courses. This was an annoying though not a serious complication.

Following the administration of twenty three courses of nitrogen mustard therapy, thirteen patients developed maculopapular markedly pruritic skin lesions over the trunk and extremities. The lesions appeared from one to six weeks after treatment. Morphologically the eruption first appeared as pink, maculopapular lesions 2 to 5 mm in diameter. They later became hemorrhagic in some cases. Throughout the development of the eruption and during its course, pruritis was marked. The lesions usually persisted for many weeks but were relatively few in number. In some cases the eruption was scattered diffusely over the body, in others it was confined to one or more extremities or to a single area of the trunk. Grossly the lesions were similar to those which occasionally are seen as skin manifestations of Hodgkin's disease. Microscopically, however, biopsy material obtained from three patients studied was described as vesiculobullous lesions presumably resulting from vascular damage. The walls of the surrounding vessels showed marked degeneration. In addition to the maculopapular lesions described five of the thirty one patients in this series developed the classic lesions of herpes zoster.

The most serious complication following nitrogen mustard therapy in this series resulted from bone marrow and lymphoid tissue destruction. Blood studies considered adequate and suitable for hematologic evaluation were done following thirty five courses of therapy in twenty six patients. A leucopenia below 2000 white cells per cubic millimeter of blood was recorded following twenty two courses. In fifteen the white blood count dropped below 1000. The lymphocytes were the first cellular elements to reflect the toxic effect of the material. A decrease in the number of lymphocytes was observed as early as twenty four hours after the first dose of nitrogen mustard in some cases. Before the institution of HN<sub>2</sub> therapy an absolute lymphopenia (below twenty per cent) was apparent in all of the patients studied. Twenty five had less than 10 per cent and seventeen had less than 5 per cent lymphocytes before treatment. In the latter group (less than 5 per cent lymphocytes) a significant decrease in the number of circulating lymphocytes following therapy was obviously difficult to measure. Fourteen of the sixteen treatment courses given to patients whose blood contained more than 5 per cent lymphocytes before therapy demonstrated a significant drop in those cells within one to five days after the first dose was given. In twenty three of the thirty four courses subsequent blood studies revealed a return to the previous lymphocyte level or to a more nearly normal level. In nine courses three weeks after therapy 20 per cent of the circulating white blood cells were lymphocytes a significant increase compared with the level before therapy. Four courses were followed by an immediate increase in lymphocytes. Two of these four experienced satisfactory remissions (Courses 17 and 42).

In addition to the lymphopenia present before therapy, in thirty-four of thirty-five instances an absolute monocytosis and an increased monocyte-lymphocyte ratio were found. The monocyte-lymphocyte ratio returned to normal and was accompanied by a satisfactory remission after fourteen of thirty-four courses. In eleven courses followed by no remission, the monocyte-lymphocyte ratio remained abnormal. When an alteration in the monocyte-lymphocyte ratio followed therapy, it consisted of both an increase in the total number of lymphocytes and a decrease in the number of monocytes. A direct correlation between variations in the monocyte-lymphocyte ratio and variation in the activity of the disease appeared to be present in twenty-five of thirty-four courses (74 per cent).

At the time when the leucopenia was most severe, approximately three weeks after therapy, all white blood cell elements were numerically reduced in equal proportion. Thrombocytopenia, when present, usually appeared a few days later than the leucopenia. A thrombocytopenia of less than 100,000 developed following seventeen of the thirty-four courses in which hematologic studies were made, thirteen patients had platelet counts below 50,000 and six below 10,000. Many of these patients developed petechiae, in one there was bleeding from the mucous membranes.

Twenty-four of the thirty-four treatment courses (71 per cent) followed hematologically demonstrated an increased reticulocyte count, as high as 10.8 per cent in one case, during exacerbations of the disease previous to nitrogen mustard therapy and during subsequent recrudescence of the disease. This reticulocytosis existed independent of the presence or absence of anemia. Following twenty-three of twenty-eight courses studied, the reticulocyte count dropped within two weeks following  $\text{HN}_2$  therapy, to 0.0 in six instances, it remained above normal at its lowest point following nineteen courses (68 per cent). The most marked anemia was noted in most cases three to four weeks after  $\text{HN}_2$  therapy and was recorded below 2.0 million red cells after eight of thirty-five treatment courses. The degree of anemia observed after therapy was dependent both on the red blood count before therapy and on the number of whole blood transfusions given the patient following therapy. A normal number of red blood cells present before treatment rarely was followed by a decrease of more than one to one and one-half million after nitrogen mustard. When the red blood cell level was low before therapy, a profound anemia frequently developed after  $\text{HN}_2$ . Whole blood transfusions were adequate for maintenance preceding marrow regeneration.

Serial sternal marrow studies were made in ten cases. Three of these revealed increases in reticulum cells, monocytes, and plasma cells before therapy. In five cases a left shift in the erythroid series was seen before treatment but there was no significant quantitative alteration in any of the normal cell types. All ten marrow samples studied demonstrated a moderate to marked hypoplasia of all cellular elements after treatment with  $\text{HN}_2$ . The diminution in marrow elements was apparent to some extent by the time therapy was completed five days after the first dose. Hypoplasia became more marked during

the subsequent two weeks and in most cases aplasia followed. Only fibroblasts and fat cells remained. After the period of aplasia, a normal regenerative process occurred with the appearance of young erythroid and myeloid forms in increasing numbers. Within five to six weeks after therapy, when the peripheral blood picture was approaching normal a hyperplasia and left shift of erythroid and myeloid elements and megakaryocytes were seen.

Serial blood sedimentation rates were determined following thirteen courses. A direct relation between clinical benefit and improvement in sedimentation rate was observed in eight of thirteen patients studied (62 per cent). The administration of folic acid in doses of 30 to 50 mg daily during and after approximately one half of the thirty-four courses observed did not seem to alter the cytotoxic effects of mustard therapy. Whole blood transfusions similarly did not alleviate the toxic depression of the marrow nor did they aid in maintaining a normal peripheral white blood cell or platelet level. All patients with a leucopenia below 2000 cells were given penicillin until the white blood count rose above this level. In three cases there was secondary infection directly associated with the granulocytopenia; the infection was controlled by penicillin in each case.

Both C. E. (Case 12) and G. T. (Case 14) whose histories are summarized below exhibited extensive damage of the hematopoietic system with return to a normal equilibrium in one and a partial return in the other. The latter patient (Case 14) experienced a longer and more complete remission than did the former (Case 12). However, in Courses 9, 12, and 14 (Cases 5, 7, and 9) illustrated in Table I, clinical remissions of two, five, and two months respectively were observed in patients who had only minimal evidence of toxic damage to the blood and lymphoid systems.

CASE 12—C. E., a 35-year-old white woman entered University Hospital during March 1947 complaining of epigastric pain, weight loss, weakness, and cough. The illness began during the third trimester of the patient's last pregnancy during the fall of 1944 when she noticed a swelling in the left side of the neck. A few weeks later the skin became yellow and pruritic. About three months after the uncomplicated full-term delivery of a normal female child, the patient was referred to this clinic because of persisting cervical adenopathy. After a left supraclavicular lymph node biopsy in April 1945 the diagnosis of Hodgkin's disease was made. The patient then received roentgen therapy over the right and left cervical regions and the mediastinum. During the fall of 1945 she received further radiation over the axillae, mediastinum, and epigastrium. She felt well until two months before remission when she developed epigastric pain which was more pronounced after eating. She stated that solid foods seemed "to get stuck" before reaching the stomach causing pain which lasted more than an hour. These symptoms progressed and were accompanied by cough, weakness, and weight loss.

Physical examination revealed a pale thin individual with cervical and axillary non-tender, firm, discrete nodes. Roentgen ray studies of the esophagus demonstrated an obstruction just behind the bifurcation of the trachea. A calcified inferior bifurcation node was visualized and roentgen ray findings suggested the possibility of ulceration into the esophagus. Following the administration of 25 mg of bis (β-chloroethyl) amine the patient became nauseated and vomited. Six days later roentgen ray examination revealed less evidence of obstruction and stenosis of the esophagus. For approximately two months the patient felt well and was able to eat solid foods without pain or difficulty. At that time dysphagia returned, became progressively more severe, and was accompanied by cough.

Physical examination revealed a widening of the mediastinum to the right. A second course of 22.5 mg of nitrogen mustard was given. During the week after HN therapy the patient noticed her cough becoming more severe with swallowing and reported that she had coughed up food particles. A diagnosis of esophageal bronchial fistula was made and tubal suction via the esophagus was instituted. Fluoroscopic examination confirmed the diagnosis of fistula. The patient had a pruritic maculopapular eruption over the extremities and chest at this time.

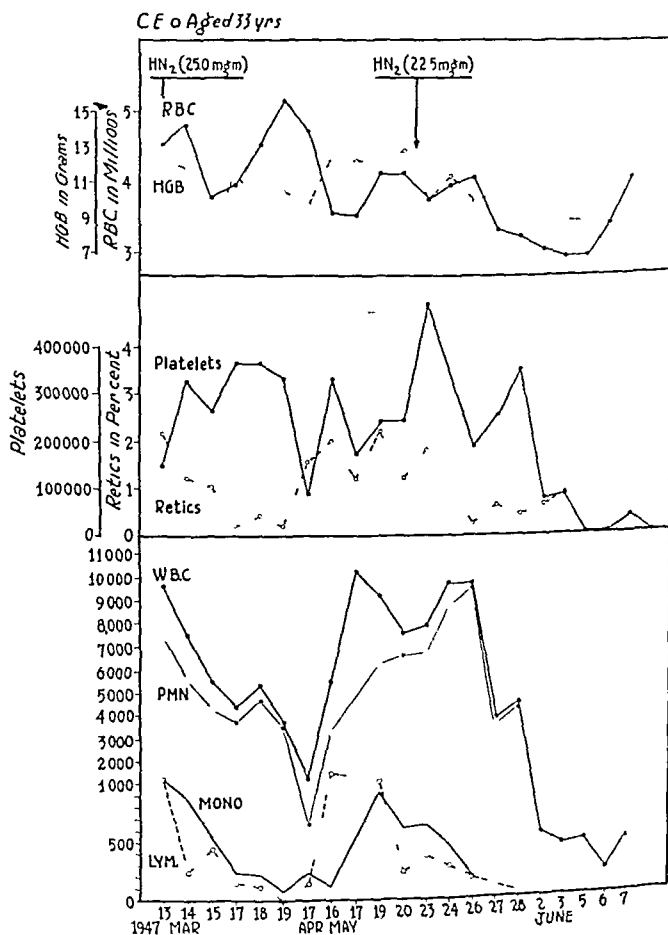
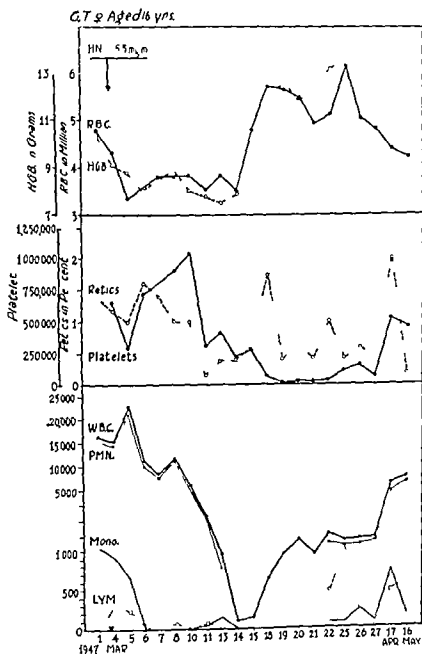


Fig 1

In spite of whole blood transfusions, glucose infusions, and penicillin, the condition of the patient worsened and she expired on the twenty third hospital day, three months after the first course of nitrogen mustard. The skin eruption became more extensive and severe about one week after the second course of mustard therapy. The anatomic findings at autopsy were (1) Bronchoesophageal fistula, (2) necrotic ulceration of the esophagus, (3) mediastinal lymphadenitis, (4) pulmonary infarction and emphysema, (5) fibrinous pleuritis, (6) multiple gastric ulcers with mucosal hemorrhage. The hematologic findings in this case are illustrated in Fig 1.

CASE 14—G T, a 16 year old white girl, was admitted to the University Hospital February, 1947, complaining of weakness, enlarged glands, itching of the skin, and cough. In 1944 the patient noticed increasing weakness, easy fatigability, and enlarged left cervical

nodes. A diagnosis of Hodgkin's disease was made following study of a lymph node biopsy section and the patient was referred to this clinic. Following biopsy the patient received multiple courses of roentgen therapy over the mediastinum, lung, abdomen and cervical and inguinal region. The patient noticed no peripheral adenopathy during subsequent exacerbations but returned for roentgen ray treatment repeatedly because of recurrence of the weakness and weight loss. Six months before admission for HN therapy the patient and she had severe generalized pruritis which continued until admission. One month before admission she developed a cough productive of thick yellow white sputum. Pain in the substernal region accompanied the cough. On admission fever, night sweat, occasional chill, productive cough, and weakness were described.



Fig

Emaciation, dark brown pigmentation of the skin and a papular eruption over the entire body with excoriations were present. There was no significant adenopathy. Roentgen ray studies revealed a right pleural effusion with apparent involvement of the right upper lobe lung parenchyma by Hodgkin's process.

The patient received five daily doses of bis ( $\beta$ -chloroethyl) amine hydrochloride (2.0 mg) with no untoward reaction. Ten days after the first dose roentgen studies revealed a diminution in the pleural effusion and increased aeration in the right lung apex. The temperature 10.2 to 10.3 before therapy was observed to be 10.5 on the first day of therapy. Two days after the first dose of nitrogen mustard the temperature was 10.0 and varied

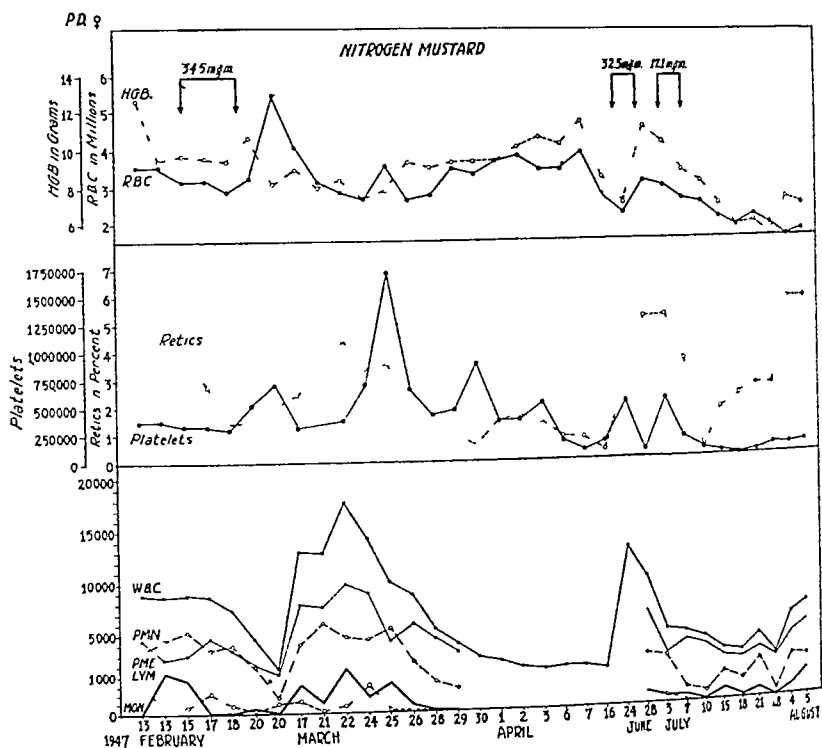


Fig 3

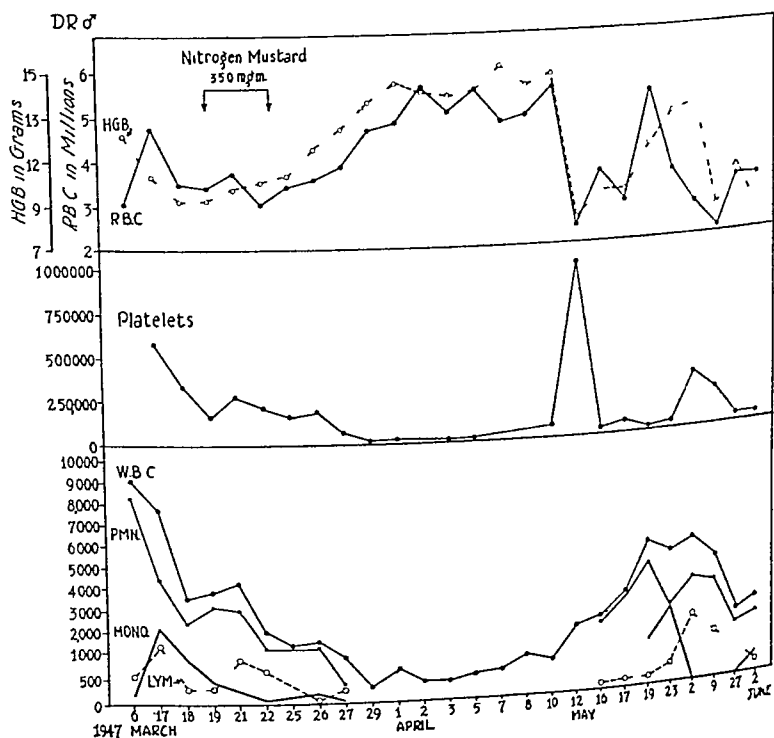


Fig 4

between 98.0 and 101 until seventeen days after the beginning of therapy at which time it returned to normal. The hematologic changes are illustrated in Fig. 2. The patient received 30,000 units of penicillin every three hours from the second day of treatment until discharge and 50 mg. folic acid orally every day after the twelfth day following the first dose of nitrogen mustard. She received four transfusions of 450 cc. of whole blood during the second week after treatment. Pruritis and the papular eruption became less noticeable and were absent when the patient was discharged twenty four days after the beginning of treatment. At the time of discharge the brown skin pigmentation had almost disappeared. Appetite, sleep and general well being were improved. The severity of the cough had greatly decreased and only a small amount of thin clear sputum was obtained. Two weeks after discharge the patient returned feeling well except for a recurrence of the skin eruption with pruritis accompanied by lesions similar to those of herpes zoster. Although the herpetiform lesions disappeared in three to four weeks, the papular pruritic eruption persisted intermittently until a second course of nitrogen mustard was given six months later following a recurrence of fever and a progression of the pulmonary lesions.

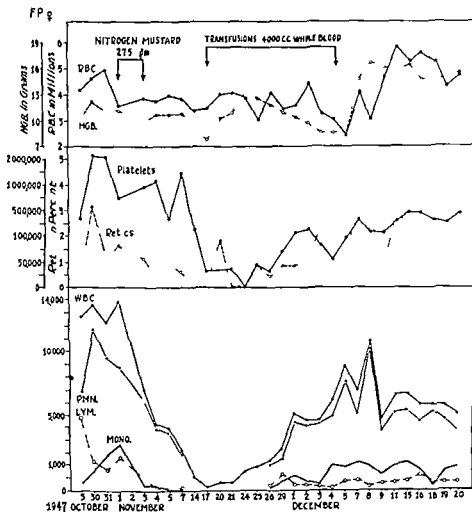


Fig. 5

CASE 31—F P a 29 year old white married woman was first diagnosed as having chronic lymphatic leucemia on the basis of a supraclavicular lymph node biopsy nine years before admission. For five years she received roentgen therapy over multiple areas of recurrent adenopathy. During most of that time she was able to maintain normal activity. Four years before admission to the hospital a second lymph node biopsy gave a diagnosis of Hodgkin's disease and this diagnosis was reinforced by a review of the old slides. Since then the patient had continued to receive occasional roentgen ray treatments. During the six months preceding this admission she noted progressive weakness, dry cough, intermittent afternoon fever, substernal chest pain, and pain over the right inferior angle of the scapula. Roentgen ray studies revealed an enlargement of the mediastinal shadow which was inter-

pieted as being due to adenopathy. The patient was given a total of 1,200 roentgens in over each of three fields: anterior and posterior mediastinum, and right lateral chest. The latter therapy did not bring about relief of symptoms. On admission to the hospital the patient also had complained of a sense of fullness in the epigastrium and pain in the right upper quadrant for minutes to hours following the ingestion of any type of food.

Roentgen ray studies of the gastrointestinal tract, gall bladder, and kidneys demonstrated a distortion suggesting adenopathy in the right lumbar and celiac axis nodes. The gall bladder could not be visualized. Basal metabolic rate on admission was plus 3 per cent, blood urea nitrogen was 11.5. Prothrombin was 86.5 per cent, brom-sulfaleim, 10 per cent in both specimens, direct van den Bergh, 0.2, indirect, 0.75. Total protein was 6.0, albumin 3.75, and globulin 3.00. The serial hematologic studies are illustrated in Fig. 5.

The patient was given two doses of methyl bis ( $\beta$  chloroethyl) amine hydrochloride of 110 mg. on two successive days and 55 mg. on the third day. The patient experienced nausea and vomiting during and for one day after the course of therapy. The pain disappeared during the first week after treatment. A cholecystogram ten days after the first dose revealed a normally functioning gall bladder. The patient was discharged eleven days after the first dose of HN, apparently improved in every respect.

Six days after discharge the patient was readmitted, having felt well until three days after discharge. At this time she experienced fever, chills, malaise, nausea, and vomiting. Physical examination revealed an ulceration of the gingiva and petechiae on the soft palate. The abdomen was diffusely tender. The oral temperature was 102° F. The white blood count was 150 and the red blood count was 3.36 million, no platelets or reticulocytes were seen. The sternal marrow was aplastic, only fibroblasts, fat and plasma cells were found. The prothrombin was 44 per cent of normal, total protein 6.00 Gm. per cent, albumin 3.42 Gm. per cent, globulin 2.58 Gm. per cent. There was 40 mg. per cent albuminuria and a blood urea nitrogen of 31.5.

The patient was given 50,000 units of penicillin every three hours but continued to have a septic temperature. No new physical signs developed until five days after admission when a rapidly progressive jaundice appeared. At this time the van den Bergh was indirect 13.2, direct 10.35, cephalin was 1 plus, total protein 6.75, albumin 2.37, and globulin 3.38. Thrombocytopenia was 20. During the course of this admission 3,500 cc. of whole blood were given without untoward reaction. Streptomycin (4 Gm. daily) was started on the fourth hospital day. The patient became distended on the seventh hospital day and a Miller Abbott tube was inserted. On the following day a right lower lobe pneumonia and atelectasis became manifest. Ten days after admission a mass was palpated in the right lower quadrant. A barium enema demonstrated a perforation of the posterior aspect of the cecum with escape of the barium into the retroperitoneal space. An incision and drainage of the retrocecal retroperitoneal abscess were done. The temperature returned to normal within three days after surgery and the patient began to improve. *Escherichia coli* and staphylococci were obtained on culture of the abscess fluid.

The van den Bergh, blood protein, prothrombin, and lung findings returned to normal within one month after the abscess drainage and at that time the drainage tube was removed. Priodax roentgen ray studies revealed closure of the abscess cavity. The cecal perforation could not be demonstrated two weeks after surgical intervention.

#### DISCUSSION

Remissions of one to ten months have been observed following HN therapy in the series of cases presented in this report. There appear to be several types of patient who may obtain benefit from this form of therapy. First, nitrofurantoin mustard has produced remissions in the clinical course both of patients who had never been given roentgen therapy and in patients who had previously received beneficial effects from roentgen radiation. Second, HN has been used



successfully in individuals who had widely disseminated Hodgkin's disease with severe systemic intoxication who were therefore unsuitable subjects for radiation therapy. Third, a regression of the disease after nitrogen mustard therapy has been observed in cases in which recent roentgen treatment over involved areas failed to produce beneficial results. Finally, an indirect benefit apparently was obtained in selected patients who previously had been considered roentgen ray resistant and who demonstrated no apparent improvement after nitrogen mustard therapy. In these patients an apparent resensitization to roentgen rays was established.

One of the most consistent clinical signs of an exacerbation of Hodgkin's disease in the present series was the presence of fever either Pel Ebsen or septic in type. As exemplified by G. T., Case 14 a decrease in temperature during or within a few days after therapy was observed sufficiently often to justify the use of this observation as an early index of the efficacy of the treatment. Relief of pain, observed in patients G. T. and F. P. (Cases 14 and 31) was also an early manifestation of a remission. Viciously pruritic skin lesions an annoying counterpart of many cases of Hodgkin's disease disappeared shortly after HN therapy in G. T. (Case 14). Objective signs of a clinical remission are exemplified by the case report of G. T. In the presence of extensive pulmonary involvement HN therapy was followed by increased aeration of the lung, a diminished amount of sputum and a decreased cough. Another objective change was observed in F. P. (Case 31) in whom the gall bladder, nonfunctioning before treatment was reported to be normal after HN therapy. The symptoms suggestive of gall bladder disease before therapy also disappeared. Skin pigmentation, a sign occasionally encountered in Hodgkin's disease disappeared within a few days after HN therapy in a few cases (Case 14). In Cases 14 and 31 a regression of the disease process was demonstrated in organs other than those usually associated with the lymphoid system. The toxic reactions to the nitrogen mustard therapy were fairly consistent for example, nausea and vomiting occurred as an immediate toxic effect following each dose of HN in 78 per cent of the courses given. The thrombophlebitis observed at the site of intravenous injection of this medication was an annoying but minor complication. The causal factor in the development of skin lesions in a number of patients after HN therapy remains to be determined. It cannot be stated whether this was a cutaneous spread of Hodgkin's disease after nitrogen mustard or a toxic effect resulting from local or systemic vascular damage. The profound leucopenia and thrombocytopenia observed after HN therapy reflects the extensive toxic damage to the hematopoietic system. The fact that the lymphocytes were the first cellular elements to be decreased in the peripheral blood suggests that the lymphocyte is the most rapidly destroyed of the formed elements of the blood. That the destruction of blood cell elements occurs as a result of a central toxic effect as well as a peripheral one is demonstrated by the concomitant destructive hypoplasia and aplasia of the bone marrow observed during the period when the peripheral count is lowest. The appearance of hyperplasia of the bone marrow and increased numbers of young

forms of myeloid and erythroid elements as well as megakaryocytes while the peripheral cellular elements are increasing during the recovery phase indicates the duration and the temporary nature of this toxic effect on the blood cell forming precursors of the hematopoietic system

The most consistent hematologic abnormalities in Hodgkin's disease before therapy are lymphopenia, monocytosis, and reversed monocyte-lymphocyte ratio. The subsequent regeneration of the lymphoid tissues and return to normal of the circulating lymphocytes after therapy reestablishes a more nearly normal monocyte-lymphocyte ratio. In this series of patients the return of the monocyte-lymphocyte ratio toward normal was the most consistent laboratory finding associated with a clinical remission.

An exacerbation of Hodgkin's disease in these cases was also reflected to some extent by an absolute reticulocytosis before therapy regardless of the presence or absence of anemia. The alterations in the reticulocyte count accompanying the bone marrow changes after  $\text{HN}_2$  therapy prohibit its use as a prognostic index of the results of treatment.

In considering patients for nitrogen mustard therapy, it should be noted that the danger of the toxic effect of  $\text{HN}_2$  on the bone marrow is minimized by two factors. One of these is the ability of antibacterial chemotherapeutic agents to alleviate the danger of infection resulting from temporary agranulocytosis and the other is the tremendous capacity of hematopoietic tissue to regenerate despite extensive damage by  $\text{HN}_2$ . Similarly, evidences of bone marrow involvement by Hodgkin's disease before therapy with accompanying anemia, leucopenia, and thrombocytopenia do not constitute an absolute contraindication to  $\text{HN}_2$ .

The fact that no consistent relationship was observed in some cases following nitrogen mustard therapy between the appearance of a clinical remission and the amount of hematopoietic and lymphoid tissue damage as measured by peripheral blood and sternal marrow changes indicates that hematopoietic tissue damage is not necessarily a criterion of the benefit to the patient resulting from this treatment. Two patients (Cases 11 and 29) in whom a satisfactory regression of the disease was observed not only failed to present gross evidence of hematopoietic damage on study of the peripheral blood but actually were observed to have an immediate increase in circulating lymphocytes following therapy.

The case reports presented described some unusual complications which might be considered as toxic reactions following treatment because of their occurrence shortly after nitrogen mustard therapy. Treatment with  $\text{HN}_2$  was followed in one case by a perforation of the esophagus and esophageal and gastric mucosal ulcerations, and in another by a cecal perforation, gastric and duodenal ulcers were observed in a third. One patient developed marked jaundice and evidences of severe liver damage shortly after treatment, findings which returned to normal a few weeks later. It cannot be determined whether any or all of these changes are the direct result of the action of nitrogen mustard or are a secondary manifestation of the destruction of active Hodgkin-

tissue. If the former is true, the destructive effect on the gastrointestinal mucosa of experimental animals by toxic doses of nitrogen mustards may also be observed in some cases at therapeutic dose levels in human beings.

Nitrogen mustard appears to be a valuable adjunct to the therapy of Hodgkin's disease although it does not replace roentgen therapy. A comparison of previously recorded results following roentgen radiation with those following the selective use of both roentgen radiation and nitrogen mustard therapy suggests that the combination of therapeutic agents may offer greater hope for increased life expectancy than either used alone.

#### SUMMARY

Thirty one cases of Hodgkin's disease were treated with a total of forty four courses of methyl bis ( $\beta$  chloroethyl) amine hydrochloride.

Beneficial results were observed in twenty patients receiving twenty four courses. Indirectly, three other patients benefited through an apparent resensitization to roentgen rays. Improvement was characterized in most instances by an immediate disappearance of fever, itching and pain. Brownish pigmentation of the skin was observed to decrease in several cases as did Hodgkin's skin lesions, splenomegaly, hepatomegaly, and adenopathy.

The toxic effects of HIN on hematopoietic and lymphoid tissues as reflected in peripheral blood and sternal marrow changes are described and discussed. These changes are contrasted with alterations in hematologic equilibria observed during exacerbations of Hodgkin's disease. Lymphopenia with a reversed monocyte lymphocyte ratio is frequently observed during the active phase of Hodgkin's disease. A regeneration of lymphocytes and return of the monocyte lymphocyte ratio toward normal is the most consistent laboratory finding associated with a clinical remission. An absolute reticulocytosis has been observed in many cases during the active phase of Hodgkin's disease before therapy regardless of the presence or absence of anemia. It is observed that no apparent relationship exists in selected cases between the amount of measurable damage to the hematopoietic and lymphoid tissues and the occurrence of clinical remissions. Bone marrow hypoplasia proceeding to aplasia and followed in every instance by complete regeneration to the previous level and in some cases to a more normal level within a few weeks after therapy was observed. Bone marrow involvement by the Hodgkin's disease process before therapy is not considered a contraindication to HN therapy.

An immediate toxic reaction of nausea and vomiting was observed consistently after HN therapy. Case reports have been presented describing unusual complications which followed HN therapy. These include ulceration and perforation of the mucosa of the gastrointestinal tract. Skin lesions macroscopically similar to those frequently seen in Hodgkin's disease appeared in an unusually large number of cases following HIN therapy.

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# THE EFFECT OF LIVER EXTRACT AND VITAMIN B<sub>12</sub> ON THE MUCOUS MEMBRANE LESIONS OF MACROCYTIC ANEMIA

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FOR more than twenty years we have been studying the mucous membrane lesions of persons with macrocytic anemia and have observed that they usually are relieved by liver extract and Ventriculin. A great opportunity to study more carefully the pathogenesis of these lesions appeared with the advent of pteroylglutamic acid (folie acid) in 1945<sup>1</sup> and 5 methyl methyl (thymine) in 1946<sup>2</sup> two pure chemical compounds which are effective in producing a hematologic response in certain types of macrocytic anemia.

As soon as it had been demonstrated<sup>3</sup> that synthetic folie acid and synthetic thymine were effective in producing a hemopoietic and clinical response in persons with pernicious anemia and related anemias<sup>4,5</sup> additional studies on these substances were planned. These studies were directed toward answering the following questions: What clinical syndromes are affected by these chemical compounds? What is the relation of the structure of the molecule of these compounds to their antianemic properties? What are the relative clinical and hematologic effects of these substances as contrasted with each other and as contrasted with liver extract? How well do these substances maintain patients with pernicious anemia, nutritional macrocytic anemia and tropical sprue? As a partial answer to this last query we (and many others) during the past two and one half years have found that folie acid maintains the blood levels in persons with pernicious anemia just as well as liver extract but that it does not offer a complete treatment in most cases since it does not prevent or relieve subacute combined degeneration of the spinal cord.<sup>6</sup> It appears from our studies that folie acid is preferable to liver extract in the maintenance of persons with nutritional macrocytic anemia and tropical sprue.<sup>7</sup> We have found that thymine, like folie acid is not a complete treatment in most cases of pernicious anemia since it neither prevents nor relieves the subacute combined degeneration of the spinal cord although it does maintain the blood levels very well.<sup>8</sup> Unpublished observations show that massive doses of thymine are as effective as liver extract or folie acid both clinically and hematologically in maintaining persons with nutritional macrocytic anemia and tropical sprue. The very large dose required, however, makes thymine impractical as a therapeutic agent.

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The folie acid was furnished by Dr Stanton M Hardy of Lederle Laboratories Inc the  
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In our two and one-half years of study, the administration of either folic acid or thymine did not result in healing the very severe mucous membrane lesions in cases of pernicious anemia. In every instance the administration of liver extract was followed by prompt improvement. This improvement lasted for varying degrees of time after the cessation of liver extract therapy. The earliest relapse occurred one month after therapy was discontinued, and several patients had had no recurrences twelve months after therapy was discontinued. The great majority had a recurrence from within one to nine months after the last injection.

Three of the patients were uncooperative and, feeling very much better, would return to work and not wish treatment again. When they relapsed they returned to us. These three patients had, at intervals, treatment with massive doses of thymine, parenteral liver extract, and oral administration of folic acid. In addition two of them had treatment with vitamin B<sub>12</sub>, the newest vitamin to be isolated<sup>9, 10</sup> and found to be effective in producing a clinical and hematologic response in persons with Addisonian pernicious anemia, nutritional macrocytic anemia, and tropical sprue<sup>11-13</sup>. The fiery redness and intense pain of the mucous membrane lesions were relieved at least temporarily, in these two patients. A case history which is representative of the patients with severe lesions follows.

J. B., a 48 year old white man. A diagnosis of pernicious anemia was first made in May, 1944. At that time he complained of general weakness, dyspnea and palpitation on exertion, soreness of mouth and tongue, swelling of feet and legs, and paresthesias of the extremities. The tongue was smooth (severe atrophy of papillae) and red, the gums were swollen and injected, and scars were present at the angles of the mouth. Sensory changes suggestive of peripheral neuritis were noted. The initial blood values were: red blood cell count, 1.25 million, hemoglobin, 5.1 Gm (33 per cent), reticulocytes, 18 per cent, white blood cell count, 3,250, packed cell volume, 16, mean corpuscular volume, 128, mean corpuscular hemoglobin, 40.8, and mean corpuscular hemoglobin concentration, 31.8. Many megaloblasts were present in the aspirated sternal marrow. Other pertinent laboratory data were: achlorhydria and achylia after histamine stimulation, slightly elevated icteric index, and negative gastrointestinal x-ray studies. Therapy with a small amount of an experimental liver fraction resulted in partial relief clinically and hematologically.

Within a period of six months the anemia and associated symptoms relapsed. The symptoms and physical findings were essentially the same as noted on the first occasion. The blood counts were: red blood cells, 1.39 million, hemoglobin 4.4 Gm, and reticulocytes 42 per cent. Therapy with commercial liver extract (Reticulogen) induced a remission rapidly. A peak reticulocyte count of 37.3 per cent was obtained on the sixth day of therapy. Five seven days after therapy was started the red blood cell count was 5.08 million and hemoglobin was 13.1 grams. Clinical improvement was just as remarkable. The signs and symptoms of glossitis and stomatitis subsided rapidly, and regrowth of lingual papillae was observed.

After an interval of nine months without therapy the patient was observed a third time in severe relapse in September, 1945. In addition to the symptoms and physical signs of anemia per se, there were severe glossitis and stomatitis and moderately severe paresthesias and physical signs of peripheral neuritis. The initial red blood cell count was 1.04 million and hemoglobin was 7.1 grams. The patient was given 100 mg of folic acid daily in mouth for twenty days. A peak reticulocyte count of 19.2 per cent was obtained on the fifth day of therapy. Sixty five days after folic acid was started the red blood cell count was 4.97 million and hemoglobin was 12.5 grams. Symptoms and physical signs of glossitis and stomatitis subsided entirely within ten days, and on the patient's release from the hospital the neurological examination was negative.

A fourth relapse developed within five months after the folic acid therapy was discontinued. The symptoms and physical findings in March, 1946, were essentially the same as noted on the three previous occasions except that there was evidence of progression of the peripheral neuritis. The blood counts were: red blood cells, 1.55 million, hemoglobin 6.2 Gm (40 per cent), white blood cells 3050, reticulocytes 0.3 per cent, packed cell volume 20, mean corpuscular volume, 129, mean corpuscular hemoglobin 40 and mean corpuscular hemoglobin concentration 31. Therapy consisted of 5 methyl uracil (thymine) 0.6 Gm daily by mouth for nineteen days. A peak reticulocyte count of 16 per cent was obtained on the eleventh day of therapy. Twenty-one days after therapy was started the red blood cell count was 2.39 million and hemoglobin was 8.9 Gm, an increase of about one half million red blood cells and 3.1 Gm of hemoglobin in three weeks time. Although studies of sternal marrow which was aspirated just after the peak of reticulocytosis revealed a normoblastic reactive stage comparable to that observed during therapy with liver extract and on another occasion with folic acid the anemia began to relapse soon after therapy was discontinued. Neither the stomatitis and glossitis nor the nervous symptoms were relieved. While the patient was taking thymine the glossitis and stomatitis became worse with swelling and increased burning soreness of mouth and tongue and ulceration and fissuring of mucosa of lower lip.

The patient left the hospital against advice, thus interrupting therapy. However on June 28, 1946, he returned to the clinic and was started on folic acid 10 mg daily by mouth. At that time symptoms and signs of glossitis and stomatitis were still present. The patient received the folic acid (10 mg four times a day) for thirteen days. A peak reticulocyte count of 26.6 per cent was obtained on the eleventh day of therapy. During the twenty-three days he was observed, the red blood cell count increased from 0.91 million to 2.19 million, and hemoglobin from 4.0 to 7.9 grams. Throughout that period glossitis and stomatitis persisted. The patient insisted on leaving the hospital again.

He was readmitted to the hospital in September 1946. Despite the persistence of severe anemia and glossitis and stomatitis he had worked regularly at a cotton mill during the previous sixty days. During that time however paresthesias recurred and became progressively worse, and disturbed locomotion developed. The patient was given liver extract intramuscularly and improved gradually, both clinically and hematologically. On December 18, 1946, the red blood cell count was 4.60 million and hemoglobin was 12.7 grams. The patient refused more therapy and returned to work.

During June 1947, the patient had an insidious onset of soreness, stinging and burning sensation of the mouth and tongue, progressive general weakness and dyspnea on exertion, and mild paresthesias of extremities. Six weeks later in July 1947 when he returned to the outpatient clinic a moderately severe glossitis and stomatitis, anemia, chronic peripheral neuritis and possibly posterior column degeneration were found. Blood counts were: red blood cells 2.00 million, hemoglobin 9.0 Gm (52 per cent), reticulocytes 0.8 per cent, white blood cells 3,200, packed cell volume, 26, mean corpuscular volume 130, mean corpuscular hemoglobin 40 and mean corpuscular hemoglobin concentration 31. A single dose 1 cc of a highly refined commercial liver extract was given intramuscularly. During the subsequent ten days the stomatitis, glossitis and cheilosis cleared rapidly and regeneration of lingual papillae began. The anemia likewise improved; the blood values obtained four weeks later were: red blood cell count 3.86 million, hemoglobin 12.2 Gm (79 per cent), reticulocytes, 0.4 per cent and white blood cell count 6,500. The patient again refused more treatment and returned to work.

During October, however, there was a gradual return of all symptoms which by the time of admission to the hospital a month later had become severe. The patient's tongue was swollen, deep dental impressions were present at the tip, all surfaces were red and the papillae apparently were severely atrophied; the buccal mucosa appeared slightly swollen and large areas were hyperemic, particularly opposite the line of closure of the teeth; tooth indentations marked the mucosa of the lower lip and several oozing fissure extended laterally externally from the left angle of the mouth.

Folic acid, 10 mg daily by mouth was started on Dec 14, 1947 and continued on March 3, 1948—a total of eighty days. It was then increased to 40 mg a day for thirty

four days, and finally to 50 mg a day for an additional period of twenty two days. At the dose of folic acid was increased to 50 mg a day, the patient received simultaneously 600 mg of niacinamide a day for seven days. Then during the next fifteen days the following vitamins (in addition to folic acid), with daily doses as indicated, were given: Vitamin A, 75,000 U.S.P. units, vitamin D, 3,000 U.S.P. units, thiamine 30 mg, riboflavin, 1 mg, niacinamide, 450 mg, ascorbic acid, 450 milligrams. While the patient was on the therapy there was considerable, but suboptimal, improvement of the anemia, but no appreciable beneficial effect, either from the folic acid alone or from the other vitamins, on the glossitis, stomatitis, and cheilosis resulted. The blood values at the beginning of therapy were: red blood cell count, 2.15 million, hemoglobin, 8.1 Gm (53 per cent), reticulocytes, 0.6 per cent, and white blood cell count, 4,900. At the conclusion of therapy (a total of 104 days) they were: red blood cell count, 4.15 million, hemoglobin, 14.2 Gm (92 per cent), reticulocytes, 2.2 per cent, and white blood cell count, 9,250. A peak reticulocyte count of 18.1 per cent was obtained on the eleventh day of folic acid therapy (10 mg four times a day).

Despite the fact that the patient's blood values were as high as we had ever seen them, the stomatitis and glossitis were worse. The fiery redness was more intense. The pain made it almost unbearable, so on April 30, 15  $\mu$ g of vitamin B<sub>12</sub> were given intramuscularly. Within twenty four hours there were equivocal signs of improvement. After forty eight hours there was definite fading of the hyperemia of the tongue and buccal mucosa. By the fifth day after the injection, the color of the mucosa of the tongue and oral cavity appeared about normal except in the most swollen areas of the lower lip, at this time the external osseous were dry and appeared to be healing. The tongue appeared less swollen, and by the thirteenth day many fine papillae were visible over the entire upper surface and the external fissures appeared completely healed. All soreness and burning sensation of the mouth, tongue, and external fissures began to subside within forty eight hours after the injection and completely disappeared within five days.

#### SUMMARY AND CONCLUSIONS

These studies show that certain patients with Addisonian pernicious anemia have severe mucous membrane lesions that are not relieved by the administration of massive doses of thymine or large doses of folic acid. These severe mucous membrane lesions are characterized by a fiery red appearance and, in most instances, excruciating pain to the patients. These severe lesions have been seen only in patients with pernicious anemia, that is to say, patients who had gastric achlorhydria and achylia, and in each instance these lesions have occurred only in people who had subacute combined degeneration of the spinal cord. There seems to be a close clinical association between the gastric defect, the severe mucous membrane lesions, and the degeneration of the posterior and lateral columns of the spinal cord in these particular patients.

Without exception, the patients in our experience, as illustrated by the case history, have benefited from liver extract injections, and two of them were given vitamin B<sub>12</sub> with similar relief for at least two weeks' time. These findings support our previous contention that neither thymine nor folic acid is a complete treatment for persons with pernicious anemia, whereas, in contrast, parenteral liver extract is.

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# THE EFFECT OF 2, 3-DITHIOPROPANOL (BAL) ON GOLD TOXICITY IN RATS

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GOLD salts have been used in the treatment of rheumatoid arthritis since they were introduced by Forestier<sup>1</sup> in 1929. Toxicity sometimes occurring with the use of gold salts has been an important factor limiting the more general use of this form of therapy.

Treatment of toxicity due to gold for the most part has been unsuccessful. 2,3-Dithiopropanol (BAL) has been reported<sup>2</sup> by a group of investigators to be successful in counteracting arsenic and mercury poisoning. These investigators made no report on the effect of BAL against gold toxicity.

Because of the value of BAL in the treatment of intoxication from other heavy metals, it seemed appropriate to study the effect of BAL in the prevention and treatment of toxicity due to gold. Clinical investigations will be reviewed separately, it is the purpose of this paper to report the effect of BAL in prophylaxis and therapy of the experimental gold toxicity in albino rats.

## EXPERIMENTAL

Albino rats averaging 160 grams in weight were used as experimental animals. Gold sodium thiosulfate,  $\text{Na}_2\text{Au}(\text{S}_2\text{O}_3)_2$ , in a 37½ per cent aqueous solution was the gold salt used in every instance. BAL always was injected in doses of 0.0375 cc, which is ten times the average dose recommended for the treatment of acute arsenic or mercury poisoning in human beings†. All injections of gold salt and BAL were made intramuscularly. The experimental animals were divided into three groups of twelve. Each of the rats in Group A was injected daily for seven days with an amount of gold sodium thiosulfate containing 18 mg of gold. An injection of BAL was made into six rats of this group on the fifth and sixth experimental days. Each rat in Group B received 47 mg of gold daily for four days. On the third and fourth experimental days, BAL was injected twice daily into half of the animals in this group. Each rat in Group C received 38 mg of gold daily for seven days. BAL was injected into six of these animals twice daily on the first three days of the experiment and once daily on the fourth through the seventh day. At the end of each experiment the animals were killed and the organs were carefully examined.

## RESULTS

All rats in Group A showed no change in general appearance, but they were less active and had diminished appetites. In behavior no differences were noted between the animals which received BAL and those that did not. One rat in the control group died on the third day, all others were killed on the seventh experimental day. Sections of the kidneys of the rats treated with BAL, in

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‡The distributors of BAL (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) recommend injections of 25 mg per kilogram body weight to be given several times daily for several days.

TABLE I CONDENSED RESULTS OF EXPERIMENT

RATS	GOLD INJECTED	BAL INJECTED	KIDNEY PATHOLOGY	REMARKS
Group A (1) 6	18 mg daily for 7 days	0.0375 cc twice daily for 2 days starting on fifth day	Moderate tubular degeneration with deposits of gold in tubules	Movements moderately slowed rats killed on seventh day of experiment
(2) 6 (control)	18 mg daily for 7 days	None	Same	Same
Group B (1) 6	47 mg daily for 4 days	0.0375 cc twice daily for 2 days starting on third day	Severe tubular degeneration extensive deposit of gold in tubules	Four rats died of toxic effects of gold before BAL was administered two rats from B transferred to C as substitutes four rats died at end of third day all rats were listless had poor appetites ruffled fur and pale eyes and tails
(2) 6 (control)	47 mg daily for 4 days	None	Same	Four remaining control rats were listless had poor appetite and pale eyes and tails rats killed at end of fourth day
Group C (1) 6	38 mg daily for 7 days	Starting on first day 0.037 cc twice daily for 3 days then once daily for 4 days	Only moderate tubular degeneration no deposits of gold salts in tubules	Rats appeared well good appetite normal fur no pallor animals killed on twenty second day for examination
(2) 6 (control)	38 mg daily for 7 days	None	Severe tubular degeneration with deposits of gold in tubules	Rats listless inactive had poor appetite and were pale from second day, two rats died on second day, 2 on third day and 1 on fifth day remaining rat killed on eighth day for examination

Group A, revealed quite normal glomeruli most tubules appeared to be entirely normal. Some tubular cells showed a moderate degree of granular degeneration in the lumina of these tubules there was frequently an aluminous precipitate. The most striking abnormality was the pigmented precipitate found in tubular cells. Dark orange and fine, red granules were seen in the epithelium of many convoluted tubules. There were some scattered deposits of golden yellow granules but these were relatively scant as compared with the fine orange red deposits. Marked congestion of the interlobular vessels was present. The collective tubules were normal, but moderate congestion of the medullary capillaries was present. The kidneys of the control rats of Group A (those animals that received no BAL) showed the same changes in the same degree as were found in the rats treated with BAL (Table I).

Of Group B, four rats died before BAL was started on the third day. The experiment was continued with four rats receiving gold and BAL and four rats served as controls. When BAL was started the entire group were listless had ruffled fur did not eat and had little color in ears and tails. The rats injected with BAL died within ninety six hours and the rats in the control group were killed on the fourth day.

In the kidneys of all animals in Group B there was severe degeneration of the epithelium in the convoluted tubules, in a patchy distribution in the epithelium and lumen. In the kidneys of rats that received no BAL, many convoluted tubules contained clumps of golden yellow granular pigment of various sizes. This pigment was found both in the epithelium and in the lumen. Some of the epithelium was necrotic and was fused to form a pale, granular, homogeneous coagulum filling and sometimes blocking the lumen. Numerous eosinophilic casts were present in the collecting tubules and in the loops of Henle. Deeply stained eosin casts blocked some collecting tubules. Among those rats that were not injected with BAL, the collecting tubules of the kidneys contained pigment also, this was usually in small, light-golden granules.

In Group C, the six rats receiving gold and BAL simultaneously appeared to be entirely normal, they ate well and were normally active during the whole experiment. They were killed on the twenty-second day in order to examine the kidneys. The control rats (which received no BAL) were listless and inactive from the second experimental day onward. Two rats of this group died at the end of the second day, two died on the third day, and one rat died on the fifth day of the experiment. The one remaining rat of this group was killed on the eighth day.

Examination of the kidneys of rats from Group C showed interesting changes. A kidney from the rat which received no BAL weighed 1.55 grams, contrasted to 1.0 gram, the average weight of one kidney from rats in this group which were injected with BAL. In the kidneys of the rats that received gold and BAL there was only moderate degeneration of epithelium in the convoluted tubules. Some of the tubules contained a small amount of albumin and occasional casts. No metallic precipitate was observed in the tissue. The kidneys of the rats in Group C that received no BAL showed severe degenerative changes, necrosis, and gold precipitate in abundance exactly as seen in the rats of Group B that received no BAL.

In none of the experimental animals was there any evidence of abscess formation in the muscle into which injections of BAL or of gold sodium thiosulfate were made.

#### DISCUSSION

In order to be certain that the abnormalities seen in the kidneys of the experimental animals were produced by the gold sodium thiosulfate injected, the kidneys of two normal, untreated rats were carefully examined. They contained no orange-red or golden yellow precipitate. Parenchymatous elements were well preserved. Some tubules showed slight vacuolar degeneration of the epithelium, and occasional hyaline casts were seen. There was considerable congestion of the glomerular and interstitial capillaries. The pigment precipitated in the kidney tissue and the renal cellular damage observed in the rats of this investigation were exactly like the changes due to gold toxicity produced in earlier studies conducted by one of us.<sup>4</sup>

In these investigations the toxicity of gold was similar to that previously described in white rats.<sup>4</sup> The chief pathologic change was in the kidneys where

nephrosis similar to that caused by some other heavy metals resulted. When the dose of gold is sufficiently large death may result quickly as was the case in the animals in Group B. Because of this the dose of gold was reduced in the studies conducted on the animals of Group C to permit sufficient time for any beneficial effects of BAL to be accomplished.

It is clear from these studies that even in large doses BAL had no therapeutic value when it was injected after extensive cellular damage already had occurred. When given simultaneously with large doses of gold sufficient to cause death in many animals of Group C BAL proved to be effective in combating toxicity from gold. Even though some renal damage then occurred it was of small amount and insufficient to cause systemic toxicity in the rats which lived and appeared well until they were killed in order to examine the tissues.

Besides the definite decrease in cellular damage a conspicuous difference in appearance of the kidneys of the rats in Group C which were treated with BAL was that no precipitate of gold or gold salt was observed in the convoluted tubules. This observation suggests that BAL combines with the gold instead of the tissue cells and keeps it in the body in a nontoxic state or eliminates it as such so as to prevent any significant cellular damage or its precipitation in renal tissue. It appears that BAL successfully competes with the host tissues for combination with circulating gold. If BAL is present in sufficient amounts before gold becomes combined with or deposited in tissues extensive cellular damage is prevented and life is sustained. The time element appears to be the most important factor in this competition for gold. Injections of BAL made soon after poisoning are effective; the same amount of BAL given later in the course of gold administration does not protect against lethal toxicity.

It appears clear therefore that to be most beneficial in human beings in combating toxicity resulting from therapeutic injections of gold salts BAL should be administered promptly when signs of toxicity are first observed; the longer the delay in use of BAL the less beneficial it likely will be. This is borne out in the clinical reports concerning BAL<sup>5,7</sup> and in unpublished clinical observations of the authors. Ragan and Boots reported BAL to be effective in protecting rats against toxicity of gold. It is impossible to compare our results with theirs for no details of dosage or time relations were published in their report.

These animal studies suggest that the manner in which BAL acts to prevent toxicity due to gold is in all respects similar to its action against arsenic and mercuric intoxication.<sup>3</sup>

#### SUMMARY

The toxicity from gold salts injected into albino rats is described. BAL had no protective effect when injected several days after toxic doses of gold were administered. When injected simultaneously with the administration of toxic doses of gold sufficient to be lethal to rats BAL prevented important toxicity; the animals lived in apparently good health. These observations indicate that BAL is an effective antidote for gold if it is given in adequate amounts sufficiently early after the administration of gold salts. These results

further suggest that to have maximal effect in human beings who have toxicity from gold salts, BAL should be given as soon as signs of toxicity are observed

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# THE EFFECT OF FEEDING PROPYLTHIOURACIL AND CHOLESTEROL ON THE BLOOD CHOLESTEROL AND ARTERIAL INTIMA IN THE RAT

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ATTEMPTS to produce arteriosclerosis in the rat have uniformly met with failure. Cholesterol feeding per se has been attempted by many without appreciably affecting either the blood cholesterol levels or the arterial intima.<sup>1</sup>

The nature of this resistance to the development of experimental atherosclerosis is unknown, and its elucidation is vital to the solution of the pathogenesis of the disease. That one of the factors involved in the process of resistance may be hormonal is suggested by the recent work of Steiner and Kendall who found that thiouracil and cholesterol fed to dogs will produce atherosclerosis, while either agent when fed alone will fail to do so. This work is highly significant because the dog does not normally develop atherosclerosis and the experimental variety of the disease has never previously been induced in this animal. Previous work has shown that the thyroid gland is involved in the entire process of experimental atherosclerosis. In rabbits thyroidectomy renders the animals far more susceptible to the cholesterolemia and atherosclerosis which follow the feeding of cholesterol.<sup>2</sup> On the other hand feeding desiccated thyroid or potassium iodide seems to protect the animals against the development of cholesterol induced lesions and lowers the blood cholesterol levels.<sup>3</sup> We have been able to confirm the protective action of desiccated thyroid in another species, the chicken.<sup>4</sup> We also have observed the synergistic effect of cholesterol and thiouracil on raising the blood cholesterol levels of the chicken.<sup>5</sup> With these considerations in mind we decided to use the antithyroid drug thiouracil in the rat in an attempt to break down the resistance of this species to the induction of atherosclerosis by cholesterol feeding.

## PROCEDURE

White rats of the Harlan strain (originally Wistar) were used. They weighed approximately 100 to 150 grams at the start of the experiment. The rats were divided into six separate groups. Group 1 was maintained on a diet of dog biscuits† and water ad libitum. Group 2 received 5 per cent cholesterol suspended in cottonseed oil mixed with ground meal‡ and tap water. Group 3 received 10 per cent cholesterol mixed with the basal diet. Groups 4, 5, and 6 received propylthiouracil§ either alone or mixed with cholesterol in

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TABLE II AVERAGE BODY WEIGHT OF DIFFERENT GROUPS IN GRAMS

GROUP	NUMBER OF WEEKS ON DIET							
	0	2	6	10	14	20	24	28
Normal	111	178	223	250	278	316	288	317
5 per cent cholesterol	142	147	221	266	314	331	329	352
10 per cent cholesterol	135	155	239	282	312	279	345	364
Propylthiouracil	153	151	191	199	215	224	196	243
5 per cent cholesterol propylthiouracil	147	138	191	188	189	185	158	220
10 per cent cholesterol propylthiouracil	145	163	170	169	165	150	156	225

\*Five animals only

†Four animals only

mean weight of 139 grams. The normal control animals gained steadily in weight and had reached a peak weight of 328 grams at the conclusion of the experiment. The 5 per cent cholesterol animals gained steadily in weight and were somewhat heavier than the normal controls, weighing 404 grams. The groups which received propylthiouracil either alone or in combination with cholesterol were far lighter than any of the control groups. Thus the propylthiouracil group weighed 257 grams at the conclusion of the experiment and the 5 per cent and 10 per cent cholesterol-propylthiouracil groups 160 and 165 grams respectively. This correlates well with the figures obtained for food intake.

*Blood Cholesterol Levels*—The cholesterol values for the normal controls showed no significant changes throughout the course of the experiment. The highest and lowest average values recorded were 53.5 and 95 mg per cent respectively with a mean of 74.1 mg per cent. There was no trend toward an increase of the blood cholesterol levels with increasing age. The 5 per cent cholesterol fed group showed a slight rise in the blood cholesterol level by the fourth week of feeding. By the eighth week of feeding the blood cholesterol level had risen to an average value of 136 mg per cent and this was the highest average value attained by this group. Thereafter, it fell off gradually and fluctuated, but remained consistently higher than that of the controls. The blood cholesterol levels for the 10 per cent cholesterol fed group showed a rise parallel to and slightly greater than those in the 5 per cent cholesterol fed group. The highest average value for this group was 142 mg per cent and was achieved by the eighth week of feeding.

In the propylthiouracil control group the blood cholesterol levels rose to 82.4 mg per cent after four weeks of feeding, and subsequently rose to 167 mg per cent by the eighth week. Thereafter there was a fluctuating decline with the values in this group somewhat higher than those for the 10 per cent cholesterol fed group.

The 5 per cent cholesterol-propylthiouracil group showed an early rise of blood cholesterol levels to 97.2 mg per cent after four weeks of feeding, and then a progressive rise to a high average value of 373 mg per cent after sixteen to twenty weeks of feeding. Thereafter there was a gradual decline in the blood cholesterol levels. The highest blood cholesterol level in any individual in this group was 616 mg per cent and was achieved after twenty-four



TABLE III AVERAGE BLOOD CHOLESTEROL LEVELS OF DIFFERENT GROUPS IN MILLIGRAMS PER CENT

GROUP	NUMBER OF WEEKS ON DIET								
	0	4	8	12	17	22	27	31	38
Normal	54*	54	81	66	95	80	88	65	64
5 per cent cholesterol		77	136	102	107	113	95	81	101
10 per cent cholesterol		86	142	95	130	129	113	118	92
Propylthiouracil		82	167	104	139	138	94	130	63
5 per cent cholesterol propylthiouracil		97	200	250	373	315	152	158	70
10 per cent cholesterol propylthiouracil		155	254	283	345	305	114	288	79

Average control value for all groups

weeks on the diet. The 10 per cent cholesterol propylthiouracil group showed the greatest early rise in the blood cholesterol of any of the groups with a level of 155 mg per cent after four weeks of feeding. This was almost three times the average control level. The blood cholesterol level rose steadily thereafter to 345 mg per cent after sixteen to twenty weeks of feeding and then declined gradually.

In summary then the feeding of either cholesterol or propylthiouracil alone in the dosage employed resulted in each case in a slight rise of the blood cholesterol levels above the normal. Five and ten per cent cholesterol in the diet produced rises in the blood cholesterol levels which were similar when the entire time course of the experiment is surveyed. When the cholesterol and propylthiouracil were combined in the diet the rise in blood cholesterol was significantly greater than in the preceding groups. The combination of 10 per cent cholesterol with propylthiouracil gave somewhat higher levels during the first twelve weeks of the experiment than the 5 per cent cholesterol combined with propylthiouracil. The high average values on all diets were achieved before the twenty second week of feeding and tended to decline thereafter. This correlates with a tendency on the part of the animals to failure of further weight gain, decreased food intake, and generally poor health which became apparent at this time.\* Toward the end of the experiment it was necessary to substitute ordinary Friskies for the prepared feed for a few days at a time because of the sickly appearance of the rats. Propylthiouracil alone produced a cholesterol emia equal to that produced by cholesterol alone. When cholesterol and propylthiouracil were combined in the diet the rise in the blood cholesterol was significantly greater than in the preceding groups. There was a moderate hypercholesterolemia which was approximately six times the average normal values or even ten times the control value in single animals. Increasing the cholesterol content of the propylthiouracil cholesterol mixture does not appreciably increase the cholesterolemia obtained.

*Pathologic Findings*—None of the animals showed any gross atherosclerotic lesions of the intima of the aorta or of the valves of the heart or of the major

\*It would appear then that the feeding of cholesterol alone in excess of 5 per cent of the diet does not produce any appreciably greater cholesterolemia than that produced by 5 per cent cholesterol or perhaps even lesser amounts of cholesterol in the diet.

the basal diet. The dose of propylthiouracil employed was 0.2 per cent at the start, it was increased to 0.3 per cent after fifteen weeks, and to 0.4 per cent after an additional five weeks. Propylthiouracil was dissolved and suspended in the drinking water in a concentration of 0.1 per cent. Group 4, consisting of eight rats, received the basal diet plus propylthiouracil in the dosage outlined. Group 5, consisting of twelve rats, received the basal diet plus propylthiouracil and 5 per cent cholesterol in oil. Group 6, consisting of eight rats, received the basal diet plus propylthiouracil and 10 per cent cholesterol in oil. In order to increase the food intake and to prevent scattering, milk powder and water were used as a binder and the feed was prepared in cake form. The amount of milk powder utilized amounted to approximately 20 per cent by weight of the total feed and added substantially to the protein and caloric value of the feed. The animals were weighed at intervals of three weeks and the blood cholesterol levels determined at that time. Two animals were chosen at random from Group 1 for cholesterol determinations and four animals from each of the other groups with the exception of Group 5, from which six animals were chosen at random for the cholesterol determinations. Blood was obtained from the tail vein, and the Schoenheimer-Sperry technique<sup>7</sup> was used for total cholesterol determination. Food and water consumption data were collected in each group at intervals of three weeks throughout the experiment. All animals were autopsied and all organs examined. Special attention was paid to the heart and aorta which were removed en bloc, slit open with fine scissors and carefully examined for evidence of atheroma. Hematoxylin and eosin stained sections were made only of those aortas which appeared to show gross evidence of atheroma.

## RESULTS

*Food Intake*—Data in Table I indicate that the normal rat consumed 41 to 42 Gm. of food in twenty-four hours. The water consumption during the same period was 27 to 42 c.c. per rat. The food consumption of the 5 and 10 per cent cholesterol fed rats approximately equalled that of the controls, with intakes of 20 to 39 and 20 to 47 Gm. per rat per day respectively. The food intake for these rats was somewhat lower than for the control group, with a more marked discrepancy in the 10 per cent cholesterol fed group. The animals receiving propylthiouracil, either alone or in combination with cholesterol, had a lower food intake than either the normal controls or the cholesterol controls. The intake for the propylthiouracil group ranged from 20 to 28 Gm. per rat per day of food and 11 to 30 c.c. of water. The intake of the 5 per cent cholesterol-propylthiouracil group was 14 to 21 Gm. per rat per day and 14 to 30 c.c. per rat per day, and of the 10 per cent cholesterol-propylthiouracil group 16 to 19 Gm. per rat per day and 13 to 20 c.c. per rat per day. It is apparent that the intake of food and water in the two latter groups was approximately half that of the control groups.

*Body Weight*—The data are shown in Table II. At the commencement of the experiment, the animals weighed between 111 and 153 grams, with a

TABLE I. FOOD AND WATER CONSUMED BY PAT DURING A TWENTY-FOUR HOUR TEST PERIOD

	5/1/47		5/23/47		6/13/47		7/3/47		8/6/47		9/3/47		10/10/47	
	FOOD (GM)	WATER (CC)	FOOD (GM)	WATER (CC)	FOOD (GM)	WATER (CC)	FOOD (GM)	WATER (CC)	FOOD (GM)	WATER (CC)	FOOD (GM)	WATER (CC)	FOOD (GM)	WATER (CC)
Normal	24	32	29	35	31	37	26	29	27	43	42	23	37	38
5 per cent cholesterol	20	24	28	24	2	-0	34	32	39	39	32	29	40	19
10 per cent cholesterol	20	18	25	22	24	20	39	24	22	39	47	18	31	27
Propylthiouracil	20	21	24	19	24	-0	3	11	12	30	23	15	26	13
5 per cent cholesterol														
Propylthiouracil	20	30	20	22	16	1	1	26	14	19	20	14	36	31
10 per cent cholesterol														
Propylthiouracil	18	13	13	16	11	-0	19	13	16	13	16	13	30	21

TABLE II AVERAGE BODY WEIGHT OF DIFFERENT GROUPS IN GRAMS

GROUP	NUMBER OF WEEKS ON DIET								
	0	2	6	10	14	20	24	28	32
Normal	111	178	223	250	278	316	288	317	303
5 per cent cholesterol	142	147	221	266	314	331	329	352	404
10 per cent cholesterol	135	155	239	282	312	279	345	364	400
Propylthiouracil	153	151	191	199	215	224	196	243	260
5 per cent cholesterol propylthiouracil	147	138	191	188	189	185	158	220	161
10 per cent cholesterol propylthiouracil	145	163	170	169	165	150	156	225	161

\*Five animals only

†Four animals only

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10 per cent cholesterol		86	142	95	130	129	113	118	92
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It would appear then that the feeding of cholesterol alone in excess of 5 per cent of the diet does not produce any appreciably greater cholesterolemia than that produced by 5 per cent cholesterol or perhaps even lesser amounts of cholesterol in the diet.

arterial trunks Fatty liver was a common finding in most of the animals which received either cholesterol or thiouracil alone, or the combination None of the control rats showed evidence of fatty liver

#### DISCUSSION

The feeding of diets containing cholesterol in high concentration, alone or combined with propylthiouracil, failed to produce atherosclerosis in the rat The diets employed resulted in rises in the blood cholesterol levels,\* but the levels found did not approach those obtained by Steiner and Kendall in the dog or by ourselves in the chicken<sup>8</sup> We have been able to demonstrate in the chicken that the development of atherosclerosis is in fairly close relationship to the degree of hypercholesterolemia and its duration<sup>8</sup> We may assume that the inability to produce a hypercholesterolemia comparable to that seen in Steiner and Kendall's dogs and in our chickens is responsible *in part* for the failure of the rats to develop evidence of atheromatosis However, it must be noted that cholesterol levels similar to those obtained in the rats in this experiment and maintained for similar periods of time would produce atheromatous changes in both the rabbit and in the chicken<sup>8, 12</sup> The failure of the rats to develop atheromatosis must be attributed in part to a cholesterol metabolic mechanism different from that which prevails in rabbits and chickens

Feeding cholesterol in excess of 5 per cent of the total diet did not produce any further rises in the blood cholesterol and this may indicate that there is a threshold for the absorption of cholesterol from the gastrointestinal tract Cholesterol fed in excess of this threshold concentration is probably excreted unchanged in the feces Equally tenable hypotheses are (1) Storage of the additional cholesterol outside the blood stream, (2) endogenous destruction of the additional cholesterol, (3) diminished endogenous synthesis of cholesterol to balance the additional amount ingested, (4) excretion of the additional amount ingested via the bile and the gastrointestinal tract Only complete cholesterol balance studies can clear up this important question

Cook and McCulloch<sup>9</sup> fed rats diets containing 2 per cent cholesterol and observed a threefold elevation in the blood cholesterol levels This is comparable to the elevation we obtained by adding 5 and even 10 per cent of cholesterol to the diet Cook's balance studies on cholesterol fed rats receiving 2 per cent cholesterol indicated that 30 per cent of the ingested cholesterol could not be accounted for and that the remainder was in the carcass, liver, and feces He also calculated that the rats absorbed 0.3 to 0.4 Gm per kilogram per day of cholesterol If we consider the average daily food intake of our rats receiving 10 per cent cholesterol as 30 Gm, then they ingested 3 Gm of cholesterol per day It is probable that nine tenths of this cholesterol passed through the gastrointestinal tract without being absorbed, although proof of this awaits cholesterol balance

\*Since no pair fed controls were used in this experiment we cannot unequivocally rule out the possibility that the relative undernutrition of the thiouracil fed rats may have resulted in hypercholesterolemia Other investigators however have found that in the rat<sup>13</sup> and dog<sup>14</sup> it does not cause any change in the blood cholesterol levels Likewise chronic undernutrition in the dog<sup>14</sup> does not cause any significant change in blood cholesterol levels

studies. It is of interest that the same threshold phenomenon has been observed in the chicken, where cholesterol fed in excess of 1 per cent of the diet does not cause any further rise in the blood cholesterol level.<sup>8</sup>

The combination of cholesterol with propylthiouracil produced a synergistic rise in the blood cholesterol levels. The mechanism for this synergism is not well understood. Fleischmann and Shumacker<sup>11</sup> believe that thyroid hormone causes a shift of cholesterol from the blood into the tissues without influencing the total amount of cholesterol in the body. Conversely then thiouracil by inhibiting the thyroid hormone should arrest this procedure or perhaps reverse its direction thereby resulting in an increase of cholesterol in the blood.

It has been reported by several observers<sup>9, 10</sup> that cholesterol feeding in the rat results primarily in an increase of the combined cholesterol in the liver with only slight increases in the cholesterol content of other organs. Sperry and Stovanoff<sup>10</sup> found that in their rats which received a 1 per cent cholesterol diet as much as 50 per cent of the total body cholesterol was concentrated in the liver. They concluded that omnivores such as the rat did not differ appreciably from the herbivores in their ability to absorb cholesterol and deposit it in the tissues, but that they differed markedly with respect to cholesterol deposition in the arteries.

Thus, while thiouracil fed to the dog diminished the resistance of that animal to cholesterol induced atherosclerosis it failed to do so in the rat. Two possible reasons for this may be considered: (1) We did not use sufficient amounts of propylthiouracil to really disturb the thyroid mechanism (however in the rat it is difficult to give much greater doses of propylthiouracil than those employed here without causing toxic symptoms to appear), and (2) in the rat other as yet unknown factors may be involved in this resistance phenomenon which were not affected by the conditions of this experiment.

It would appear then that new avenues of approach must be opened. A combination of endocrine inhibition involving more than one of the endocrine organs concerned with lipid metabolism may shed further light on this problem.

#### SUMMARY

The feeding of diets containing either cholesterol alone in concentrations of 5 to 10 per cent or propylthiouracil alone in concentrations of 0.5 to 0.6 per cent, or both combined, for periods up to thirty eight weeks failed to produce vascular lesions of the arteriosclerotic variety in rats.

The feeding of cholesterol or propylthiouracil alone produced a cholesterol emia of two to three times the normal values. Combining both gave average values approximately six times normal average values and as much as ten times normal for individual rats.

Cholesterol in excess of 5 per cent did not produce any further rise in the blood cholesterol levels. Results of feeding 5 and 10 per cent cholesterol combined with propylthiouracil compared closely with one another.

Propylthiouracil did not succeed in breaking down the rat's resistance to cholesterol-induced atherosclerosis. The resistance to atherosclerosis in this animal is probably dependent on other factors in addition to the thyroid gland and its secretions.

We are indebted to Dr. L. N. Katz for his suggestions in pursuing this study and to the other members of the department for their invaluable help in executing the studies.

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## LABORATORY METHODS

### USE OF SPLENIC INFUSION AS A BASE FOR GROWING CERTAIN PATHOGENIC MICROORGANISMS

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NUMEROUS methods for isolating and/or preserving certain microorganisms in their original state for long periods of time have been the object of many investigations

Lloyd<sup>1</sup> showed that certain accessory growth factors were essential to primary isolation of *Neisseria intracellularis* and that subsequent to isolation, provided an abundant supply of free amino acid was present the organisms gradually developed a change in metabolism becoming increasingly independent of the so called vitamin content in the medium. The results of Cole and Lloyd<sup>2</sup> showed that the growth of *Neisseria gonorrhoea* was considerably dependent upon hydrogen ion concentration (the mean optimum being pH 7.6) concentration of amino acids, and the presence of certain accessory growth factors or vitamins

Numerous investigators have reported various media supplying accessory growth factors. Worth,<sup>3</sup> in an effort to provide a universal medium for promoting growth and keeping fastidious microorganisms viable used a nutrient gelatin containing peptone and sodium chloride. Spray<sup>4</sup> found that certain fastidious organisms grew well upon a hormone gelatin agar to which sodium caseinate and soluble starch were added. James found that a 20 per cent infusion of the quahaug, a species of hard shelled clam was an excellent source of growth promoting factor for many of the more fastidious microorganisms

The lyophile and cryochem methods of Flossdorf and Mudd<sup>5</sup> employing the principle of freezing and rapid dehydration in vacuo are recognized as superior for the preservation of cultures but the costs of elaborate equipment and the time and effort expended in maintaining large collections of microorganisms may be prohibitive. The maintenance of bacteria by overlaying the culture with sterile paraffin oil as recommended by Morton and Pulaski<sup>6</sup> has advantages but necessitates the use of special selective media for primary growth

For general and ready use any culture medium that is simple to prepare that can be sterilized in the autoclave without subsequent addition of body fluids and in which the most fastidious microorganisms will grow luxuriantly and remain viable would be ideal. The employment of beef spleen as a growth promoting factor has fulfilled this need in our own investigations

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Spleen as a source of growth-promoting factor was suggested by the work of Cole and Lloyd<sup>2</sup> who used a tryptic digest of casein with extract of pig pancreas, which they named tryptamine, and an infusion of beef spleen for growing *N gonorrhoeae*. It was further suggested by Zinsser and Bayne Jones<sup>1</sup> who state that Neufeld found that pneumococci remained viable in the desiccated spleens of infected mice for periods far exceeding those on selected media.

To further determine the value of spleen as a factor in growth and viability, the following experiments were conducted.

#### PROCEDURE

Cultures of eleven of the more fastidious organisms for viability studies were made on various media consisting of splenic infusion agar, splenic infusion gelatin, beef infusion agar, and beef infusion gelatin in varying amounts, with and without the addition of vitamin B<sub>1</sub>. Splenic infusion base was prepared in a manner similar to the preparation of beef infusion base, the usual amount of peptone and sodium chloride being added to filtered minced beef spleen infused overnight. The pH was adjusted to 7.6 before autoclaving. Ten per cent gelatin was added to the splenic infusion medium and both 10 and 15 per cent gelatin to the beef infusion medium. When vitamin B<sub>1</sub> was employed in the medium it was sterilized by Seitz filtration and subsequently added aseptically in a 10 per cent concentration.

The organisms used were three strains of streptococci (*Streptococcus pyogenes*, *Streptococcus viridans*, *Streptococcus anhemolyticus*), three of pneumococci (*Diplococcus pneumoniae* Types 1, 2, and 3), and one each of *Neisseria intracellularis*, *Neisseria gonorrhoeae*, *Hemophilus pertussis*, *Corynebacterium diphtheriae*, and *Hemophilus influenzae*.

All media were tubed in plastic screw-top vials, 120 by 20 mm, to insure the preservation of moisture during incubation and storage. Prior to inoculation of experimental media, three successive transfers of each organism were made twenty-four hours apart upon selective media for each species.

Inoculations were done with a standard 3 mm loop from an emulsion of the test organism in mammalian Ringer's solution made to a density corresponding to McFarland nephelometer tube No. 8. This was to insure uniformity of the inoculum of each organism and to prevent a carry-over of nutrient material from the selective medium on which the organisms were growing.

Five tubes of each type of medium were inoculated with the various organisms. After forty-eight hours of incubation at 37° C, growth of each was recorded, the tubes were sealed and placed in the refrigerator at 10° C, with the exception of *N intracellularis*, *N gonorrhoeae*, and *H influenzae*. The first two were kept in the incubator at 37° C, the tubes of the latter were immersed in cold water and placed in a dark cupboard at 20° C. Growth in each instance was indicated as negative (-), good (+), and heavy (++) Table I shows the record of growth on various media with appropriate symbols.

With the exceptions of *H. influenzae* and *H. pertussis* splenic infusion media in every instance, both with and without vitamin B<sub>1</sub>, gave rise to excellent initial growth, which was superior to that on beef infusion media.

TABLE I PROLIFERATION OF GROWTH OF ORGANISMS AFTER FORTY EIGHT HOURS OF INCUBATION

ORGANISM	SPLenic INFUSION BASE				BEEF INFUSION BASE			
	SIV	SIIV	SIG	SIGV	BIIV	BIIGV	BIIG15	BIIG15V
<i>Str. pyogenes</i>	++	++	+	+	+	+	+	+
<i>Str. viridans</i>	++	++	+	+	++	+	++	++
<i>Str. anhemolyticus</i>	++	++	+	++	++	+	++	++
<i>D. pneumoniae</i> 1	+	+	+	+	+	+	+	+
<i>D. pneumoniae</i> 2	++	++	+	+	++	++	++	++
<i>D. pneumoniae</i> 3	++	++	+	+	+	++	++	++
<i>N. gonorrhoeae</i>	++	++	-	-	-	+	+	+
<i>N. intracellularis</i>	++	++	+	-	-	+	+	+
<i>H. influenzae</i>	-	-	-	-	-	+	+	+
<i>H. pertussis</i>	-	-	-	-	-	-	-	-
<i>C. diphtheriae</i>	++	++	++	++	++	+	++	++

SIV Splenic infusion agar, SIIV splenic infusion agar plus vitamin B<sub>1</sub>, SIG splenic infusion agar plus 10 per cent vitamin B<sub>1</sub>, SIGV splenic infusion agar plus 15 per cent vitamin B<sub>1</sub>, BIIV beef infusion agar plus vitamin B<sub>1</sub>, BIIGV beef infusion agar plus 10 per cent vitamin B<sub>1</sub>, BIIG15 beef infusion agar plus 15 per cent vitamin B<sub>1</sub>.

Transfers from each type of medium were made at intervals of one and one half, three, six, nine and twelve months to determine viability. They were made from suspension in Ringer's solution from the growth in the sealed stored tubes and also by direct transfer to selective media.

Additional studies on splenic medium were made to determine its value as a base for blood agar, its ability to support growth of the pathogenic clostridia and its use in primary isolation of organisms. For this purpose Petri dish streak cultures of *Str. viridans*, *D. Pneumoniae*, *Str. pyogenes*, *C. diphtheriae* and *N. intracellularis* were made on plain splenic agar, splenic blood agar and plain blood agar in order to compare growth and colonial characteristics. Pathogenic clostridia, *Cl. botulinum*, *Cl. tetani* and *Cl. novyi* were grown in deep slabs of splenic infusion agar and broth to determine whether these media would warrant their use for growing anaerobes. Studies on splenic infusion agar were made to evaluate its use in primary isolation of organisms from sputa, nasal washings, and throat cultures. Microorganisms so isolated were grown on splenic infusion agar and vaccines were prepared from these.

## RESULTS

*Str. pyogenes* was found viable on all media except beef infusion agar plus 10 per cent plus vitamin B<sub>1</sub>. *Str. viridans* was living only on beef infusion agar plus 15 per cent and beef infusion agar plus 15 per cent plus vitamin B<sub>1</sub>, and *Str. anhemolyticus* was living on all the cultural media at the end of one and one half months. *Str. anhemolyticus* was living on all media and *Str. pyogenes* only on the splenic infusion agar both with and without vitamin B<sub>1</sub> at the end of twelve months, whereas *Str. viridans* failed to remain alive longer than three months at which time it was living only on beef infusion agar plus 15 per cent. See Table II.

It will be noted from these results that the strain of *Sti anhemolyticus* employed survived on all types of media for the entire period of the experiment (twelve months) and that *Sti pyogenes* was maintained on splenic infusion agar for a period of one year, but that *Sti viridans* did not survive on any form of splenic media

TABLE II SURVIVAL TIME OF ALL ORGANISMS TESTED ON SPLENIC AND ON BEEF INFUSION MEDIA AT VARIOUS INTERVALS

ORGANISMS LIVING AT END OF (MO)	ORGANISM	SPLENIC INFUSION MEDIA				BEEF INFUSION MEDIA			
		SIA	SLAV	SIG	SIGV	BIIV	BIGV	BIG15	BIG12V
1½	Str pyogenes	+	+	+	+	+	+	+	+
	Str viridans	-	-	-	-	-	-	+	+
	Str anhemolyticus	+	+	+	+	+	+	+	+
	D pneumoniae 1	+	+	+	+	+	-	+	+
	D pneumoniae 2	+	+	+	+	+	+	+	+
	D pneumoniae 3	+	+	+	+	+	+	+	+
	N gonorrhoeae	+	+	-	-	+	-	+	-
	N intracellularis	+	+	+	-	+	-	-	-
	H influenzae	-	-	-	-	-	-	+	-
	H pertussis	-	-	-	-	-	-	-	-
	C diphtheriae	+	+	+	+	+	+	-	+
3	Str pyogenes	+	+	-	+	-	-	+	+
	Str viridans	-	-	-	-	-	-	+	+
	Str anhemolyticus	+	+	+	+	+	+	+	+
	D pneumoniae 1	-	-	+	+	-	-	+	+
	D pneumoniae 2	-	-	+	+	-	-	+	+
	D pneumoniae 3	-	-	+	+	-	+	+	+
	C diphtheriae	+	+	-	+	-	-	+	+
6	Str pyogenes	+	+	-	-	-	-	+	+
	Str anhemolyticus	+	+	+	+	+	+	+	+
	D pneumoniae 1	-	-	+	+	-	-	+	+
	D pneumoniae 2	-	-	+	+	-	-	+	+
	D pneumoniae 3	-	-	+	+	-	+	+	+
	C diphtheriae	+	+	-	-	-	-	+	+
9	Str pyogenes	+	+	-	-	-	-	-	+
	Str anhemolyticus	+	+	+	+	+	+	+	+
	D pneumoniae 3	-	-	-	+	-	-	-	-
	C diphtheriae	+	+	-	-	-	-	-	-
12	Str pyogenes	+	+	-	-	-	-	-	-
	Str anhemolyticus	+	+	+	+	+	+	+	+

The three strains of *D pneumoniae* survived as follow At the end of one and one half months, all strains, with the exception of *D pneumoniae* 1 on beef infusion gelatin 10 per cent plus vitamin B<sub>1</sub>, were alive on all media At the end of six months, survival was recorded on the splenic infusion gelatin, but not on the splenic agar By the end of nine months all cultures had died

*C diphtheriae* was viable following storage on splenic infusion agar for nine months On the other hand, similar cultures on any of the beef infusion media failed to survive *N intracellularis* and *N gonorrhoeae* were alive at one and one half months on splenic agar Both of these organisms usually require transfers every forty-eight to seventy-two hours on chocolate agar, the medium of choice for their growth Splenic medium was not conducive to the growth of *H pertussis* or *H influenzae*

Alpha hemolysis of *St. viridans* and *D. pneumoniae* and beta hemolysis of *Str. pyogenes* were readily distinguishable on splenic blood agar. Colonies of these organisms on splenic blood agar were larger than the usual pinpoint ones on plain blood agar, and the zone of hemolysis was more readily discernible

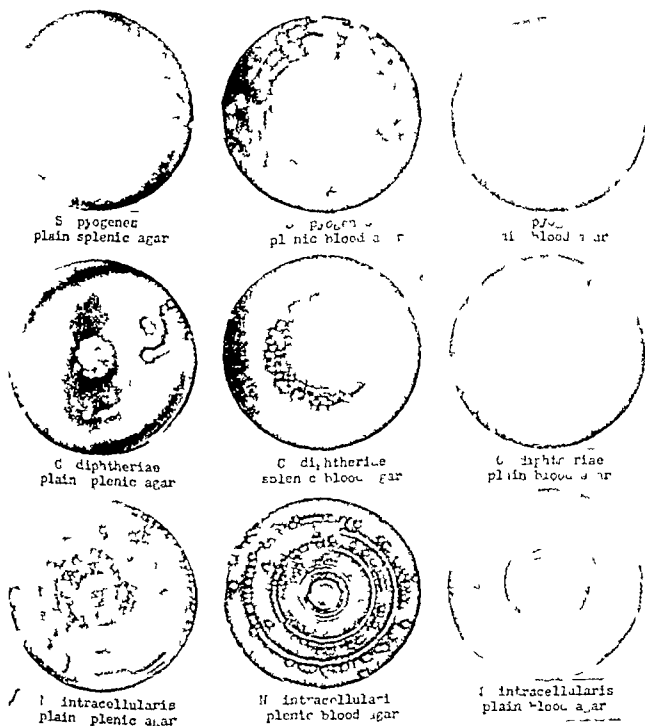


Fig. 1

Growth and hemolysis were both marked in less than twenty four hours. Fig. 1 illustrates the excellent growth of these organisms upon plain splenic and splenic blood agar, and the exceptional colony growth and hemolytic action of *Str. pyogenes* on splenic blood agar compared with that upon plain blood agar. The addition of blood to the splenic medium except for detecting hemolysis, offers no particular enhancement to the growth.

The clostridia were grown in deep stabs of splenic infusion agar and in splenic infusion broth. Growth was excellent and compared favorably with that in ground beef medium or broth to which sterile tissue had been added. Blackening of the agar and broth occurred in deep strata of the medium in a manner similar to the blackening action in brain medium and gelatin as ordinarily observed in certain of the anaerobes.

The profuse growth on splenic infusion media of the organisms studied except *H. pertussis* and *H. influenzae*, has proved excellent for primary isolation and for the preparation of certain bacterial vaccines. Plain splenic infusion agar furnishes suitable growth for laboratory teaching.

#### SUMMARY AND CONCLUSIONS

Spleen infusion as a base for agar has been used satisfactorily for maintaining cultures of certain strains of streptococci, *C. diphtheriae*, *N. intracellularis*, and *N. gonorrhoeae* over periods of time in excess of the usual limits for carrying stock cultures of these organisms.

Transfers for carrying stock cultures of most of the fastidious organisms may be made at intervals of one to two months or longer, instead of at weekly or shorter intervals.

A universal medium such as splenic agar is easily prepared, requires no subsequent addition of body fluids, and eliminates the preparation of selective media for growth of each type of the more fastidious organisms to be cultivated.

Luxuriant growth of most of the blood-loving organisms on this medium exceeds that on a selective medium in each instance. Splenic medium has proved excellent for primary isolation and the preparation of bacterial vaccines. Splenic infusion as a base for broth, agar, or gelatin has been used successfully in classroom work. Pathogenic clostridia grow well in these media.

No studies have been made to determine what growth factor or factors may be present in spleen. Apparently the amino acids in the commercial peptone present, released during bacterial development, plus growth factors from the splenic infusion provide excellent nutrient material. No observable advantage is gained through the addition of vitamin B<sub>1</sub> to splenic media. The preservation of moisture by the use of tightly sealing plastic caps makes possible storage of this medium for several months.

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# AN IMPROVED MOUNTING FOR THERMOCOUPLES FOR THE MEASUREMENT OF THE SURFACE TEMPERATURE OF THE BODY

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## INTRODUCTION

THE intensity of radiation, measured by a radiometer, provides the best index of the temperature of the outermost surface of the skin. The radiometer is an accurate instrument but is technically difficult to employ, it requires daily calibration, the conversion of electric potentials to temperature is laborious, and an observer or trained subject must hold the instrument in position for each reading. Thermocouples, on the other hand, are technically easy to use, but give inaccurate measurements of surface temperature. This is the result of the type of mounting employed. When a naked thermal junction is used, readings are affected by the temperature of the ambient air, and firm contact between the junction and the skin is difficult to maintain. When the thermocouple is protected from the air by a covering, heat loss from the skin is impeded and the readings are too high. The difference between surface temperature measurements by thermocouple and radiometer is generally  $1^{\circ}$  to  $3^{\circ}$  C.<sup>1</sup>

## MATERIALS

A thermocouple mounting was developed which allowed the skin surface temperature to be determined with much greater accuracy than previously. This mounting (Fig. 1) was made on a 1 by 3 inch rectangle of copper window screen (16 mesh, wire diameter 0.01 inch). Copper-constantan thermocouple wire\* was used, and kinking was prevented by plastic spaghetti. Insulation was retained to just beyond the point where the wires passed under the screen, and the insulated portion was lashed to the screen with thread. The naked leads were twisted together and the junction, about one-half inch in length, was soldered to the under side of the screen as indicated. The screen remained quite flexible except for the ends, which were dipped in soft solder to provide firm connections for metal snap buttons. Adjustable bands of elastic cloth were attached to these buttons and held the mounting firmly against the skin.

## RESULTS

Surface temperature measurements using these assemblies were compared with readings obtained by radiometer in four different environments. Thermocouples were fastened to the belly, chest, and thighs of nude subjects and temperatures on adjacent skin areas were determined simultaneously by both

From Medical Department Field Research Laboratory

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\*The most satisfactory wire was nylon-insulated 7 strand 36 gauge obtained from Revere Corporation of America Wallingford Conn



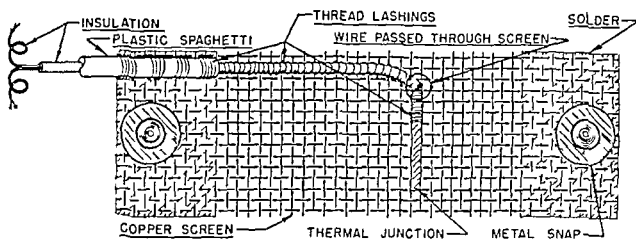


Fig 1—The thermocouple assembly

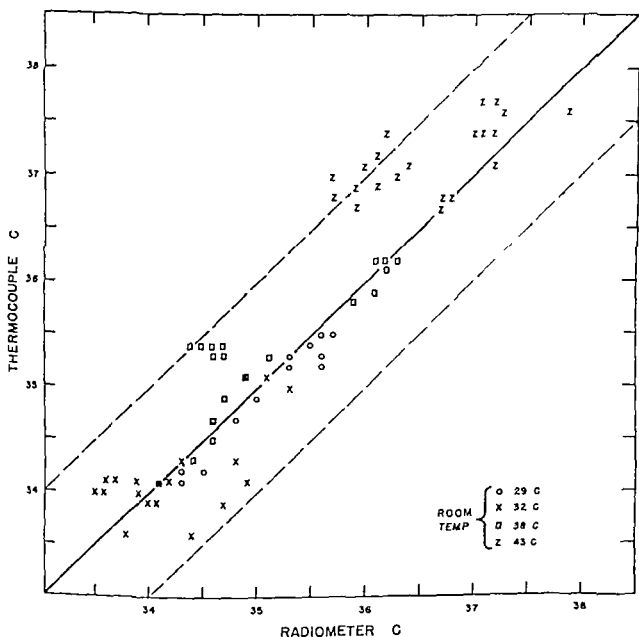


Fig 2—Comparison of simultaneous thermocouple and radiometer measurements of surface temperatures.

methods. The results are shown in Fig 2. The broken lines on each side of the central diagonal show the limits of variations of plus minus 1 C between the readings by each method and enclose 95 per cent of all measurements. It was not possible to measure temperature by both methods simultaneously on

exactly the same skin area, and, since the skin temperature may be appreciably different in immediately adjacent areas, a better comparison between the methods is made by comparing the average of a series of readings. Such averages are shown in Table I.

TABLE I COMPARISON OF THERMOCOUPLE AND RADIOMETER READINGS

ROOM TEMPERATURE (° C)	NUMBER OF VALUES	AVERAGE SURFACE TEMPERATURE (° C)		DIFFERENCE (° C)
		THERMOCOUPLE	RADIOMETER	
29	16	34.9	35.0	-0.1
32	23	34.2	34.2	0.0
38	24	35.4	35.0	+0.4
43	28	37.1	36.5	+0.6

The room temperature still affected the thermocouple readings slightly in the hot environments. The subjects were sweating profusely at these temperatures, and the difference in readings presumably resulted from impaired evaporation. The agreement between radiometer and thermocouple, however, was markedly superior under these circumstances to the agreement when either naked, covered, or partially covered thermocouples with conventional mountings were used. The screen mounting always remained firmly in place despite muscular movement or heavy sweating.

#### SUMMARY

Thermocouples mounted on copper window screen were designed for the measurement of the skin surface temperatures of human subjects. In environments ranging from 29° to 43° C the average deviation of a series of thermocouple readings from a simultaneous series of radiometer readings was -0.1° C in the coolest environment and only +0.6° C in the hottest. The assemblies were easily constructed, they always remained firmly in place and were quite sturdy.

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## STUDIES OF PANCREATIC FUNCTION

### IV A SIMPLIFIED METHOD FOR THE DETERMINATION OF SERUM LIPASE USING AQUEOUS TRIBUTYRIN AS SUBSTRATE WITH ONE HUNDRED NORMAL VALLES BY THIS METHOD

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IN 1943 Goldstein and Roe<sup>1</sup> published a procedure for estimating serum lipase in which the conditions for the enzyme action were so improved that only one hour's incubation at 37° C. was necessary. In this procedure the substrate was dispersed with bile in the digestion mixture. At that time it was observed that the substrate, tributyrin, could be satisfactorily dispersed in an aqueous medium since tributyrin is slightly soluble in water. Preliminary experiments revealed that if tributyrin is properly homogenized in an aqueous buffered digestion mixture, it is split more rapidly by blood serum than when it is emulsified with bile or bile salts. It seemed desirable to take advantage of this observation since the end point of the titration with the aqueous suspension is more easily discerned and the difficulty of preparing and titrating a bile emulsion is eliminated.

Since previous observations were made with cat serum as the source of the enzyme it was felt that the experiment should be repeated using the sera of human subjects. We repeated this work using the sera of four students all apparently in good health. The tributyrinase concentration of each sample of serum was determined in three ways: (1) using the glycerol bile emulsifying agent as reported in our previous paper; (2) substituting 5 c.c. of distilled water for the glycerol bile mixture; (3) omitting the glycerol bile and thus reducing the volume of the digestion mixture by 5 cubic centimeters. The results of this study are recorded in Table I. Our data show that human sera respond in the same way as cat sera to the omission of the glycerol bile emulsifying reagent. This study also demonstrates that it is not necessary to substitute the distilled water for the glycerol bile. The determinations were carried out under the conditions outlined in our previous paper.<sup>1</sup>

In order to be sure that this difference in the measurement of tributyrinase activity of human sera represented the limitations of bile and not a difference in the hydrogen ion concentration of the digestion mixtures caused by the presence or absence of bile it was necessary to determine the pH of the digestion mixtures before and after hydrolysis. We carried out this experiment using a pH paper method\*. The data presented in Table II demonstrate that the differences in hydrolysis are not caused by significant differences in the pH of the digestion mixtures.

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TABLE I COMPARISON OF TRIBUTYRINASE ACTIVITY OF HUMAN SERA WITH AND WITHOUT GLYCEROL BILE

DIGESTION MIXTURE	SERUM			
	1	2	3	4
With glycerol bile	45	72	37	49
Substitution of distilled water	119	166	115	135
Omission of glycerol bile	120	172	119	134

Values are expressed as cubic centimeters of 0.1N KOH per 100 cc of serum (tributyrinase units)

TABLE II pH OF THE DIGESTION MIXTURES BEFORE AND AFTER HYDROLYSIS

DIGESTION MIXTURE	BEFORE HYDROLYSIS	AFTER HYDROLYSIS
With glycerol bile	8.4	8.0
Substitution of distilled water	8.4	8.2
Omission of glycerol bile	8.4	8.2

The new method developed with the use of an aqueous tributyrin emulsion is described below

## MODIFIED METHOD FOR SERUM TRIBUTYRINASE DETERMINATION

*Reagents*—

*Substrate* Tributyrin \*

*Buffer* Dissolve 5 Gm of sodium diethylbarbiturate in distilled water and make up to 1 liter

*Calcium Acetate Solution* 20 Gm of calcium acetate, chemically pure  $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$ , are dissolved in distilled water and the solution is made up to 1 liter

*Alcohol-Ether Inactivating Mixture* To 900 cc of 95 per cent ethyl alcohol add 100 cc of ether

*Indicator* Dissolve 1 Gm of phenolphthalein in 100 cc of 95 per cent alcohol

*Standard Alkali* A 0.05N solution of potassium hydroxide is prepared

*Procedure*—Pipette 0.75 cc of tributyrin, 30 cc of the sodium diethyl barbiturate solution, and 30 cc of the calcium acetate solution into the receptacle of a hand homogenizer† and pass this mixture through the hand homogenizer three times. Then pipette 20 cc portions of the emulsion into two large test tubes, one the control and the other the experimental. Both the control and experimental tubes are placed in a constant temperature water bath at 37° C, and when the emulsions have reached this temperature 1 cc of the serum is pipetted into the experimental tube and mixed thoroughly with the emulsion by means of a stirring rod. After one hour of hydrolysis, 1 cc of serum is pipetted into the control tube and the enzyme in both tubes is inactivated by pouring each mixture into a 250 cc Erlenmeyer flask containing 100 cc of the alcohol-ether mixture. The contents of each flask are then titrated with the standard potassium hydroxide solution, using phenolphthalein as the

\*Eastman Kodak Company Rochester N Y

†Schaar and Company Chicago Ill

indicator, to the same shade of light, but definite pink color. The difference between the two titration values is a measure of the concentration of lipase in the serum. In agreement with conventional methods of designating blood values, we have expressed our results in terms of concentration of enzyme per 100 cc of serum. One tributyrinase unit is the amount of enzyme that will catalyze the hydrolysis of tributyrin with the release of 1 cc of 0.1N fatty acid in one hour at 37° C with the reagents and conditions as described.

TABLE III ELECTROMETRIC TITRATION OF SERUM TRIBUTYRINASE CONCENTRATION (RABBIT SERUM)

SERUM	pH AT POINT OF INACTIVATION	pH AT END POINT OF TITRATION	0.05 KOH (cc)	TRIBUTYRINASE UNITS
Control	9.60	10.65	0.1	—
Experimental	9.35	10.65	3.1	55

The final step in this procedure the titration can be carried out more accurately as an electrometric titration. We have found the use of a Peelman calomel glass electrode pH meter (model H 2) in conjunction with an electric stirrer very satisfactory for this titration. In the electrometric titration the end point is at pH 10.65, this corresponds to the end point of the phenolphthalein titration. The difference in the hydrogen ion concentrations at which phenolphthalein turns pink in an aqueous solution (pH 8.3) as compared with an alcohol ether medium is due to the suppression of ionization in the alcohol ether mixture. An example of the electrometric titration for determining serum tributyrinase concentration is presented in Table III.

#### SERUM TRIBUTYRINASE CONCENTRATION IN ONE HUNDRED NORMAL HUMAN SUBJECTS

Using the technique as outlined we have determined the tributyrinase concentration of the serum of one hundred persons including medical students, graduate students, and faculty members. The group consisted of eighty six men and fourteen women, all apparently in good health. The ages of the group varied roughly from 20 to 50 years, with the majority of the group being in the 20 to 30 year range. All of the samples of blood except seven were drawn in the postabsorptive state, evidence at this time indicates no significant difference in the level of serum tributyrinase before and after meals.

The results of this study of serum tributyrinase levels in one hundred healthy subjects are graphically shown in Fig. 1. The lowest value of this group was 81 tributyrinase units, while the highest concentration was 246 tributyrinase units. There were only three samples of serum with values below 100 tributyrinase units, then there was a sharp rise in the number of samples of serum with values ranging from 100 to 180 tributyrinase units. This was followed by a sharp fall in the number of samples (nine) of serum with values over 180 units. Thus 88 per cent of the samples of serum showed a tributyrinase concentration ranging from 100 to 180 units. Further analysis of these values showed the mean normal to be 146.26 the standard deviation to be 003, and the standard error to be 3.01.

Analysis of the values of serum tributyrinase when the samples were divided as to the sex of the donor gives one the impression that the serum tributyrinase of women is decidedly lower than that of men. Fig. 1 portrays this graphically. It is readily observed that the majority of female values fall between 100 and 150 units, the mean normal for the female group was 121.14, the standard deviation 19.11, and the standard error 5.11. When the male group is analyzed it is seen that the majority of values fall between 100 and

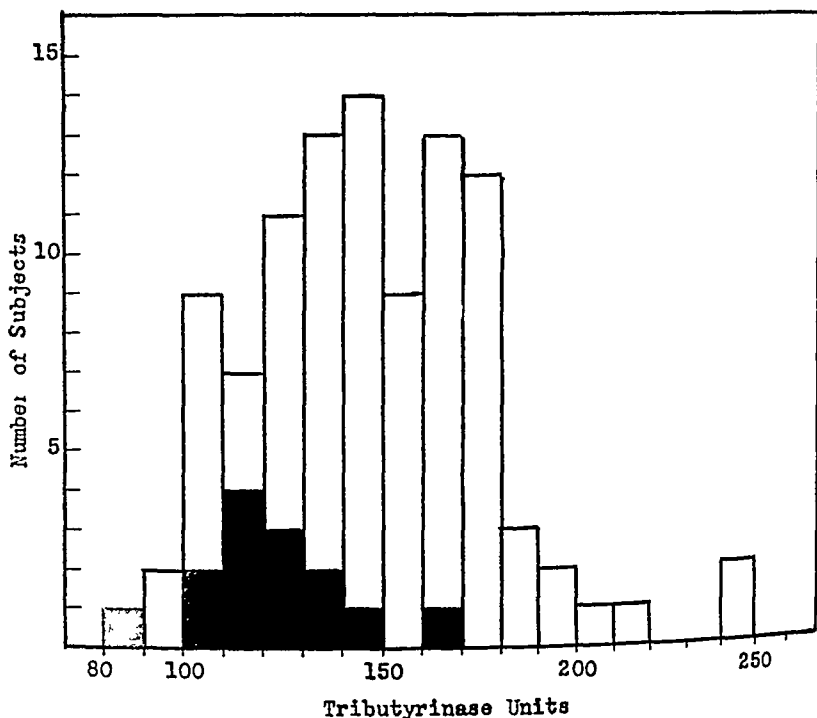


FIG. 1.—Distribution of serum tributyrinase values of one hundred normal human subjects. The height of the column represents the total number of subjects; the area in black, the number of women; and the difference between the two, the number of men.

180 units, the mean normal for the male group was 150.42, the standard deviation 29.45, and the standard error 3.18. In spite of the great discrepancy between the number of samples of serum from men (eighty-six) and those from women (fourteen), the difference between the serum tributyrinase levels of the two sexes is found to be significant when subjected to statistical analysis using the method of the difference between two means ( $t = 2.928$ , therefore  $P < 0.01$ ).

Utilizing the data further, it can be observed that the 5 per cent fiducial limits for the one hundred determinations of serum tributyrinase will be from 86.55 to 205.97 units. In general it may be inferred that our normal range more roughly will have 85 as the lower limit and 205 as the upper limit. Within this range, 97 per cent of the samples of serum have a tributyrinase concentration that can be considered to be within normal limits, 1 per cent is below the lower limit, and 2 per cent are above the upper limit.

## SUMMARY

A modification of the serum tributyrinase method eliminating the use of bile or bile salts, has been developed

The final step in the procedure the titration of the fatty acid may be carried out either with phenolphthalein as the indicator or electrometrically

The serum tributyrinase level of one hundred normal subjects has been determined

The range of tributyrinase values for the male subjects was higher than that of the female group

The normal range of serum tributyrinase levels was found to vary from 80 to 200 units

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# A CLINICAL METHOD FOR THE DETERMINATION OF HUMAN ALBUMIN BY MEANS OF A PRECIPITIN REACTION

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THE need for a rapid and accurate method for the estimation of human albumin in serum or other body fluids in the routine clinical laboratory has long been felt. Among those available methods\* which might be adapted to such a purpose are the salt fractionation method of Howe,<sup>1</sup> the methanol precipitation method of Pillemer and Hutchinson,<sup>2</sup> the immunologic method,<sup>3</sup> and electrophoretic analysis with the Tiselius apparatus.<sup>4</sup> Although several modifications<sup>5-6</sup> have been introduced into Howe's original technique, no procedure which permits a clear-cut separation of albumin and globulins has yet been devised. The chief purpose of our studies is to demonstrate that the precipitin method yields results which approach the accuracy of electrophoretic analysis, the generally accepted standard, and exceeds the other methods in speed, accuracy, and the saving of labor. Furthermore it permits an accurate determination of albumin present in body fluids in an amount too small to be determined by any other means. The results to be reported in this communication were collected for a period of more than a year from routine analyses in the laboratories of the Squibb Institute for Medical Research and the Sloan Kettering Institute for Cancer Research.

The principle of the immunologic method is based on the finding that the turbidity produced as a result of the reaction between human albumin and its homologous rabbit antiserum can be used as a measure of the precipitinogen present.

The method for the preparation of the human albumin antigen and the protocol for the immunization of rabbits have been given in a previous paper.<sup>3</sup> A few modifications which have been introduced into the procedure for the albumin determination are described in detail here, and the results of a number of albumin determinations made by the most widely used chemical methods have been included for comparison.

## METHODS

*Standardization of Pooled Antihuman-Albumin Rabbit Sera*—Each pool of antihuman-albumin rabbit sera was standardized by measuring the turbidity

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The authors are indebted to Miss Lois Hall for technical assistance in the determination of albumin by the immunologic method and to Mrs. Barbara Gotlieb for making the electrophoretic analysis.

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\*The method of Milne<sup>6</sup> was published after the collection of our data was started and therefore was not included in this series.



of the immune precipitate produced by the addition of known amounts of human albumin (ranging from 5 to 50  $\mu\text{g}$  of albumin nitrogen) to a sufficient amount of antiserum (20 ml) to insure an excess of antibody. Thus 1 per cent human albumin solution containing 1.474 mg. nitrogen per milliliter by Kjeldahl analysis was prepared by dissolving 1.0 Gm. of electrophoretically homogeneous human albumin in about 50 ml. of 0.85 per cent NaCl solution. This solution was neutralized with enough sodium bicarbonate (approximately 25 mg.) to bring the pH of the solution to 7.6 and then made up to a volume of 100 milliliters. The stock albumin solution was kept in the icebox as the standard. Thymol was added as the preservative. In order to standardize a batch of pooled sera, the standard albumin solution was further diluted quantitatively to the following concentrations expressed in micrograms of albumin nitrogen per milliliter: 2.95, 5.90, 7.37, 8.84, 10.32, 11.79, 14.74, 17.69, 20.64, and 29.48. Two milliliters of each of these albumin solutions were added to a series of Klett-Summerson tubes containing 10 ml. of 0.85 per cent saline solution. Two milliliters of the rabbit immune serum were then added to all the tubes. The reaction was allowed to proceed at room temperature for at least thirty minutes, and the turbidity of the immune precipitates\* was measured at a wave length of 420 millimicrons. A solution containing 20 ml. of the antiserum and 30 ml. of 0.85 per cent NaCl solution was used as a blank. Under these experimental conditions the antibody in the antiserum was present in such a large excess that the immune precipitate did not flocculate as large aggregates. A uniform suspension with constant and reproducible turbidity readings was obtained by gentle stirring. The turbidity readings which reached a maximum within thirty minutes were then plotted against the micrograms of human albumin nitrogen added, Fig. 1.

*Determination of Albumin in Human Serum or Plasma*—One half milliliter of human serum or plasma was diluted to 100 ml. with 0.85 per cent sodium chloride solution, and this solution was used for the determination of the total nitrogen by the micro-Kjeldahl method† and for the determination of albumin concentration. One milliliter of this solution was pipetted into two Klett-Summerson tubes each containing 20 ml. of 0.85 per cent sodium chloride solution for duplicate determinations. Two milliliters of the standardized antiserum were added to each tube. The contents in the tubes were allowed to stand at room temperature for thirty minutes or longer, and the turbidity was measured with a Klett-Summerson photoelectric colorimeter against a control tube containing 20 ml. of antiserum and 30 ml. of the saline solution. This procedure permits a technician to complete at least fifty albumin determinations in one working day.

\*If hemolyzed antihuman albumin serum was used the immune precipitate was centrifuged for ten minutes and the supernatant was removed by suction with a fine capillary attached to a vacuum, care being taken not to remove any immune precipitate. Then 5.0 ml. of a normal saline solution were added to suspend the immune precipitates and the turbidity readings were measured.

†Since we have accumulated our data, it has been found equally accurate to determine the total protein concentration by measuring the turbidity produced by 2.0 ml. of the diluted serum (1.00) and 3.0 ml. of 5 per cent trichloroacetic acid solution. The turbidity of the trichloroacetic acid precipitate bears a quantitative relationship with the total protein nitrogen determined by the Kjeldahl method.

*Calculation*—The per cent of the total nitrogen in the sample present as albumin nitrogen (see Table I) was calculated as follows. One can estimate the amount of human albumin (C) corresponding to the turbidity readings (B) from the standard curve (Fig 1). Multiplying C by the serum dilution (A)

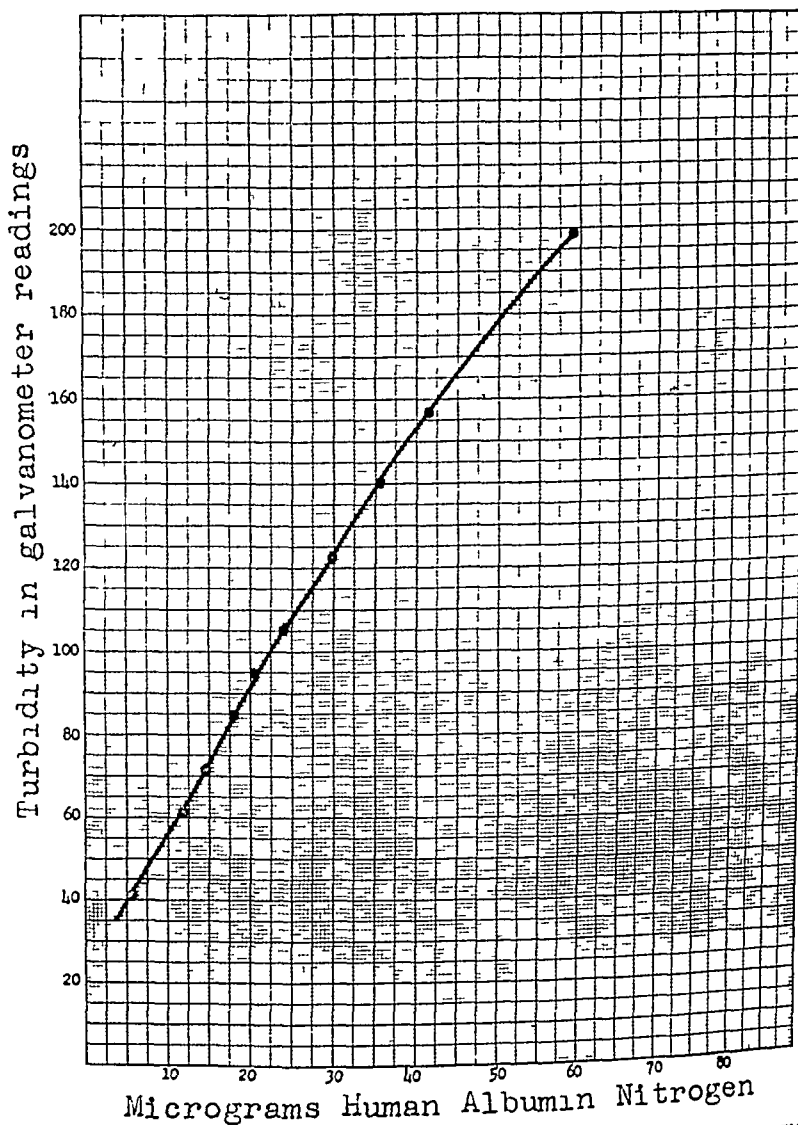


Fig 1—The relationship between the turbidity of immune precipitate and the amount of human albumin nitrogen added to 2.0 ml of its homologous antiserum

gives the total albumin nitrogen present per milliliter of the original sample (D). The ratio of D to the total nitrogen in milligrams per milliliter of the original serum (E) as determined by the Kjeldahl method or in terms of the turbidity of the trichloroacetic acid precipitate, multiplied by 100, is the per cent

TABLE I THE CALCULATION OF HUMAN ALBUMIN CONCENTRATION FROM THE TURBIDITY READINGS

SAMPLE	A DILUTION	B TURBIDITY READINGS	C ALBUMIN NITROGEN PER SAMPLE ( $\mu$ G)	D = A $\times$ C ALBUMIN NITROGEN PER ML ( $\mu$ G)	E TOTAL NITROGEN (MG/ML)	F = $\frac{D \times 100}{E}$ ALBUMIN (%)
1	1 200	142	36.5	7.30	11.98	61
1	1 400	92	19.5	7.80	11.98	65
2	1 100	139	35.0	30	9.93	35
2	1 200	87	18.0	60	9.93	36
3	1 100	105	23.5	2.5	1.00	20
3	1 200	59	11.0	2.20	12.00	18

of total nitrogen as albumin nitrogen. The results of the calculation of three samples of sera containing different percentages of albumin are given in Table I for illustration.

## RESULTS

The results of the analysis for human serum plasma albumin by different methods are presented in Table II. The data are arranged according to the total number of analyses expressed in per cent which agreed within certain limits with the electrophoretic analyses. They demonstrate that over half (58 per cent) of the samples analyzed by the immunologic method agreed within

TABLE II RESULTS OF THE DETERMINATION OF HUMAN ALBUMIN BY DIFFERENT METHODS ARRANGED ACCORDING TO THE DEVIATIONS FROM THE ELECTROPHORETIC ANALYSIS (TAKEN AS 100 PER CENT)

RANGE OF AGREEMENT* (%)	AGREEMENT (%) BETWEEN ELECTROPHORETIC ALBUMIN AND THAT DETERMINED BY		
	PRECIPITIN REACTION	SALT FRACTIONATION†	METHANOL PRECIPITATION‡
0-5	44	0	15
0-10	58	0	24
0-15	76	0	5
0-20	91	5	47
0-30	98	10	68
0-45	100	44	9
0-55	100	90	100
0-65	100	95	100
0-75	100	100	100
Total number of samples analyzed	144	22	34

In the calculation of agreement the figures obtained for albumin by the electrophoretic analysis were assumed to be 100 per cent. The analyses were done in Veronal or Veronal citrate buffer of ionic strength 0.10 and pH 8.6.

†The Howe method as modified by Robinson and co-workers was used.

‡The precipitation and filtration of the precipitates were performed in an ice bath at about 1°C.

10 per cent about three-fourths (76 per cent) agreed within 15 per cent and 91 per cent agreed within 20 per cent of the electrophoretic albumin. On the other hand, none of the results obtained by the salt fractionation method agreed within 15 per cent. 5 per cent of the samples agreed within 20 per cent and only 10 per cent agreed within 30 per cent of the electrophoretic albumin. Similarly, the distribution of the agreement between the electrophoretic and

the alcohol precipitation methods<sup>2</sup> was as follows 24 per cent of the analyses was within 10 per cent and 38 per cent was within 15 per cent of the electrophoretic albumin. The data therefore indicate that the routine determination of albumin by the immunologic method approaches the accuracy of the electrophoretic analysis. In the present comparative study the electrophoretic albumin was assumed to be correct and therefore assigned as 100 per cent. This assumption was not totally justified since several errors, as pointed out by Petermann and co-workers,<sup>7</sup> are inherent in the electrophoretic technique. However the cumulative error may amount to only a few per cent.

The analytic results also were grouped according to the per cent of electrophoretic albumin present in the sera. Within each group the agreement between analysis by the electrophoretic and other methods is recorded (see Table III), the figures obtained by the former method were taken as 100 per cent. The data demonstrate that the immunologic results agreed within a few per cent of the electrophoretic albumin for sera ranging from 20 per cent (hypoalbuminemic sera) to 60 per cent (normal sera) of the total serum proteins. In other words, this method is applicable equally to severely hypoalbuminemic sera.

TABLE III COMPARISON OF ANALYTIC RESULTS OF HUMAN ALBUMIN IN SERA CONTAINING DIFFERENT AMOUNTS OF ELECTROPHORETIC ALBUMIN (TAKEN AS 100 PER CENT)

ALBUMIN IN SERA (%)	PER CENT* OF ELECTROPHORETIC ALBUMIN FOUND BY		
	PRECIPITIN REACTION	SALT FRACTIONATION	METHANOL PRECIPITATION
20-30	101 $\pm$ 4.0	195 $\pm$ 10	-----
30-40	102 $\pm$ 3.6	114 $\pm$ 5.2	17 $\pm$ 4.1
40-50	105 $\pm$ 2.7	142 $\pm$ 3.8	121 $\pm$ 3.6
50-60	106 $\pm$ 3.8	133 $\pm$ 1.9	120 $\pm$ 4.7

\*Standard deviation

and to normal sera. On the other hand, the agreement between analysis by the electrophoretic and two other chemical methods appears to be best at a high percentage of albumin, and the discrepancy becomes larger as the per cent of albumin in the sera decreases. This situation can be explained on the basis that the so-called albumin obtained according to the fractionation methods contains not only albumin but also alpha globulins.<sup>8</sup> The per cent of alpha globulins in hypoalbuminemic sera is generally much higher than that in normal sera.<sup>8</sup>

*Application of the Precipitin Method*—The chief advantages of the immunologic method over either the chemical or electrophoretic methods lie in the small quantity of albumin necessary for each determination and in the specificity of the method. These advantages make it possible to apply this method to the estimation of albumin in fluids of clinical interest. For example, we have followed the disappearance of albumin after an intravenous administration of 75 Gm. of human albumin to a patient with metastatic melanoma. The total circulating plasma protein, as well as the total circulating albumin, was determined shortly before and at various times after injection. The results, given in

\*According to our experience it was essential to use freshly obtained serum samples for the determination with the methanol method, otherwise the results are erratic and may be off as much as 100 per cent or more. The method may be satisfactory in the hands of research chemists but is difficult to control adequately in a clinical laboratory.

Table IV, show that the injection of albumin brought about (1) an increase in total circulating plasma proteins from 168 to 217 Gm an increase of 49 Gm, and (2) an increase of albumin from 98 to 163 Gm an increase of 65 grams. The percentage of albumin was raised from 58 to 75 per cent. These effects were demonstrable on the  $3\frac{1}{4}$  hour samples. However twenty four hours after injection the total plasma proteins but not the albumin dropped to the preinjection level thus the per cent of albumin in the total serum proteins remained practically unchanged. When these studies were extended to other types of

TABLE IV DISAPPEARANCE OF ALBUMIN AFTER AN INTRAVENOUS INJECTION OF 75 GM OF HUMAN ALBUMIN

TIME AFTER INJECTION (HR.)	T O P (GM)	T C A (GM)	ALBUMIN (%)
0	168.1	—	58
$3\frac{1}{4}$	216.8	162.6	75
5	236.7	163.5	69
$7\frac{1}{4}$	196.8	140.0	71
$13\frac{1}{4}$	215.0	151.1	70
$21\frac{3}{4}$	169.9	—	—
$24\frac{1}{4}$	173.6	151.3	71

T C P Total circulating plasma proteins

T C A Total circulating albumin

For the calculation of proteins in circulation the plasma clots were determined by the method of Grogersen

cases the rates of disappearance of albumin were found to vary from one patient to another. It is to be emphasized therefore that the data presented in Table IV serve only to illustrate the usefulness of the precipitin method and not to demonstrate the rapid disappearance of albumin following albumin transfusion.

Other applications of the immunologic method may include the estimation of the albumin in the urine of nephrotic patients and the determination of albumin in the cerebrospinal fluid. The possible diagnostic significance of the latter determination was pointed out in the preliminary note of Kabat<sup>9</sup> and associates.

#### DISCUSSION

In this communication we have presented the analytic data obtained by several methods for the determination of human albumin in serum or plasma. Like other investigators, we found that the salt fractionation procedure gave the least reliable results particularly with plasma from hypoalbuminemic patients and that the methanol method is an improvement over the salt fractionation procedure. However, Bock<sup>10</sup> concluded from his study that the ammonium sulfate method yielded essentially the same results as the alcohol method. The immunologic method was found to yield results which agreed well with the electrophoretic albumin, regardless of its concentration. Besides being accurate and fast, the immunologic method requires much less material for each test and therefore permits the estimation of albumin in such physiologic fluids as spinal fluid and urine.

In a preliminary note, Kabat<sup>9</sup> and collaborators reported an immunochemical estimation of albumin. Their procedure was very similar to that published by one of us<sup>3</sup> except that the quantity of immune precipitate was measured by Kjeldahl analysis instead of by turbidity. We found that the turbidity method

is not only rapid and time saving but also gives results which are sufficiently accurate for a clinical method. It avoids the separation of the immune protein from the other proteins in the sera and the preliminary washings which are required by the Kjeldahl determinations.

#### SUMMARY

The results of routine estimation of human albumin by different methods were compared. It was found that the immunologic method appeared to be superior to the chemical methods and gave reliable results with sera containing different amounts of albumin. Because of the minute quantity of albumin required for each analysis and the specificity of the test, the immunologic method also has been found useful to estimate the excretion of albumin in the urine of nephrotic patients and to determine the amount of albumin in the spinal fluid.<sup>9</sup>

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## SYNTHETIC DIETS

### THEIR USE AS A DIAGNOSTIC PROCEDURE IN ALLERGIC DISEASE

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#### INTRODUCTION

THE evaluation of the role of ingested foods in the production of allergic symptoms is difficult and present methods of approach are frequently unsatisfactory. It is well established that the usual cutaneous and intracutaneous tests with food allergens are of little practical value in this regard for it is generally agreed that positive skin tests do not necessarily indicate clinical sensitivity.<sup>1</sup> It is equally apparent that opinion regarding the incidence and importance of foods as allergens varies widely.<sup>2-4</sup>

One of the basic principles used both in the diagnosis and treatment of allergic disease is that removal of the patient from the offending allergen is followed by remission of the symptoms which resulted from the exposure to that allergen. Therefore if the symptoms of which any patient complains are due primarily to ingested allergens and if said patient can be maintained on an intake free of offending agents the symptoms should disappear. This thesis is the basis for the use of most rotational and elimination diets. Furthermore, if patients could be maintained upon a nutrient preparation to which they could not become sensitized the question as to whether or not food factors are important etiologically in the production of symptoms in any given case could be answered finally and decisively in a short time. In an effort to take advantage of these principles various nutrient preparations of low allergenic potential have been used previously. Hill<sup>5</sup> used Amigen in combination with Dextrimaltose, olive oil, arrowroot, starch, brewers yeast and various minerals as a substitute food for milk sensitive infants with eczema. The proteins and amino acids were assumed to be nonallergenic and the author noted that the preparation was negative upon skin test in patients with a positive skin reaction for casein. In thirty-six patients to whom this preparation was given nineteen had a satisfactory result that is the food was well taken, the eczema improved and there was weight gain. In nine patients the procedure was unsatisfactory and in the remaining cases results were inconclusive. Hampton<sup>6</sup> used a similar preparation for two patients with purpura and obtained relief of symptoms in both. Olmsted, Harford and Hampton<sup>18</sup> reported the use of an enzymatic casein digest in combination with a fat (corn, olive or cottonseed oil), dextrose, minerals and gelatin for nine patients with various allergic disorders. After a seven day

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trial of the diet, they included or excluded foods as etiological factors in the production of symptoms. Glynn<sup>6</sup> mentioned the use of protein hydrolysates in infants with severe eczema, and stated that 50 per cent of his patients improved.

With such a background in mind, numerous patients have been maintained on synthetic diets at the University Hospital in an effort to evaluate more definitely the role of ingested allergens in the production of allergic symptoms.

### METHOD OF STUDY

Several formulas were used in the study of the cases which will be enumerated below. The first preparation used was Amigen, the pancreatic hydrolysate of casein, 77 per cent of which was in the form of simple amino acids and 23 per cent as short chain polypeptides. Pork or sheep pancreas was used for the enzymatic digestion. All of the known essential amino acids were present in the product, 150 Gm of which contained two thirds of the daily mineral requirement except for magnesium and potassium. A great volume of work has been done on the oral and parenteral administration of Amigen and other protein hydrolysates. It appears to have been established that Amigen will not sensitize laboratory animals to subsequent injections of skimmed milk, pancreatic extract, or Amigen itself,<sup>9, 18, 27</sup> that protein hydrolysates are absorbed from the gastrointestinal tract following oral administration,<sup>28</sup> and that excess amounts of nitrogen are not recovered from either urine or stool following oral or parenteral administration.<sup>2, 11</sup>

Amigen was prepared for serving by dissolving 100 Gm of the powder in 1 liter of hot water to which 225 Gm of cane sugar were added. This was served over cracked ice in four equal portions at regular intervals throughout the day. The mixture contained 1,200 calories and the equivalent of 75 Gm of protein. A greater quantity was occasionally used if it was thought advisable to keep the intake at higher caloric level. Some patients preferred Amigen and water without added sugar. In these instances the specified amount of carbohydrate was eaten separately as desired during the course of the day. More recently, in some cases Protolysate\* has been used in lieu of Amigen. The only essential differences between the two was that the enzymatic activity utilized in the production of Protolysate was derived from fish creca.<sup>29</sup>

The second formula used contained Amigen in combination with Dextrimaltose and olive oil†. A third preparation consisted of Amigen, dextrose, and certain synthetic vitamins‡. The general composition and caloric equivalents of each of these preparations have been compiled in Table I.

TABLE I

FORMULA	CONTENTS	PROTEIN (GM)	CARBO- HYDRATE (GM)	FATS (GM)	CALORIES PER LITER
1	Amigen (or Protolysate) 100Gm Cane sugar 225Gm Water to make 1,000cc	75	225	0	1,200
2	Mead Johnson No. 232 454Gm Water to make 1,000cc	67.5	250	85	1,942
3	Mead Johnson No. 211 454Gm Water to make 1,000cc	64	354	0	1,612

\*Mead Johnson & Company

†Mead-Johnson Laboratory Product #232 Dextrimaltose 43.95 per cent, olive oil 18.40 per cent, Amigen 20 per cent, starch 10.39 per cent, calcium gluconate 3.64 per cent, other mineral salts and vitamins 3.32 per cent (monobasic potassium phosphate, dibasic potassium phosphate, calcium hydroxide, potassium chloride, magnesium oxide, thiamin, riboflavin, niacinamide).

‡Mead-Johnson Laboratory Product #211 dextrose 77.86 per cent, Amigen 18.99 per cent, other mineral salts and vitamins 3.15 per cent (sodium chloride, calcium carbonate, dibasic calcium phosphate, dibasic potassium phosphate, ferrous sulfate, thiamin, riboflavin, ascorbic acid, citric acid).



All patients had a complete study which included history, general physical examination and routine laboratory work (serology, chest x-ray, urinalysis, complete blood count). An allergic survey followed which included an allergic history, complete cutaneous and intracutaneous skin tests in most cases, and other studies (vital capacity, cytology of exudates) if indicated. Before starting a patient on a trial of synthetic diet the possibility of allergic symptoms arising from causes other than ingestants was excluded insofar as possible by the following practices:

- (1) A period of adjustment in the hospital (four to six days) was completed to permit clearing of any symptoms which might have been the result of environmental factors.
- (2) An antidust plan was instituted in forty of the fifty-one cases. Among the other patients there was neither clinical story nor positive skin test to suggest dust sensitivity, and symptoms were those most unusually due to dust allergy.
- (3) Acute or chronic, active respiratory infection if present was treated by chemotherapy and/or antibiotics until maximal response was obtained.
- (4) Pollen sensitive patients were not evaluated on synthetic diets during the specific pollen season.

Finally, two other precautions were observed in the effort to minimize interference with the trial of diet:

- (1) Symptomatic management was simplified as much as possible so that the result of diet could be more readily evaluated. For instance effort was made to control asthma with but one symptomatic drug, so that the number of dose adjustments required served as a convenient measure of response.
- (2) Collateral therapy was eliminated during this period if possible. This was frequently difficult, particularly in the case of diffuse cutaneous disease.

If the program of synthetic diet was initiated, patients were managed thereafter as follows: Diet was continued for a period of ten days as a general rule. If there was definite remission of symptoms before the ten days had elapsed, sometimes the course was shortened. If there was equivocal response at the end of ten days, the diet occasionally was continued for a longer time. If there was no symptomatic response whatsoever in the ten days' time, dietary management of the patient was abandoned and food allergy was considered a most unlikely etiology for the patient's complaints.

When patients showed a definite remission of symptoms upon the synthetic diet, one of two courses was followed. Single food additions could be made at three- or four-day intervals if the purpose was to identify single allergens. In such cases foods held in clinical suspect were added initially. If, however, the patient's clinical condition necessitated more rapid return to an adequate natural diet, food additions were multiple, in groups of two to four, and foods thought to be innocuous were added first. Factors which conditioned this decision were the clinical condition of the patient, the severity of prior symptomatology, and the advisability of reprecipitating it.

#### CASE SELECTION

All patients selected for trial of diet in this study were experiencing daily symptoms of an incapacitating nature. They all had been surveyed as previously described and if symptoms persisted which were thought possibly to be allergic in origin, synthetic formulas were advised to aid in the evaluation of the role of ingestants in the production of their symptoms. Thus in many instances the program was used because inhalant and drug allergens had been reasonably excluded and symptoms had persisted. Despite case selection by a method which had few positive criteria, the number of patients for whom the program was advised did not increase rapidly. In 1946-1947, over an eighteen-month period, there were but fifty-one such instances. Final diagnoses in these cases have been tabulated in Table II.

TABLE II

Perennial rhinitis	1
Bronchial asthma	26
Bronchiectasis*	1
Emphysema*	2
Chronic urticaria	2
Atopic eczema	7
Chronic ulcerative colitis	3
Cephalalgia	2
Allergic colitis	1
Vascular allergy	1
Chronic conjunctivitis	1
Erythema multiforme	1
Ulcerative stomatitis	1
Periarteritis nodosa	1
Disseminated lupus†	1
Total	51

\*Admitting diagnosis was bronchial asthma

†Discharge diagnosis uncertain disseminated lupus considered the most likely Biopsy not conclusive

Multiple diagnoses were the rule in most cases, reflecting the well known tendency for allergic disease to affect more than one organ system, but the predominating symptom-complex was used for the purpose of the classification in Table II. It should be emphasized that of the fifty one patients put on the synthetic diets, forty-eight were hospitalized.

#### OBSERVATIONS

No diagnostic or therapeutic measure is of general value, however accurate or beneficial it may be, unless it can be applied readily to most patients for whom it may be indicated. It must, therefore, be admitted that these formulas were almost uniformly distasteful to patients, and those on synthetic diet programs required complete explanation initially and continued moral support throughout the trial period. In our experience, no one of these formulas was definitely more acceptable than any one of the others. However, against the initial objection on the part of most patients stands the fact that of the fifty-one to whom the diet was offered, all but three attempted the trial. Of the three patients refusing to continue the program after their initial meal, one had a vague cephalalgia later diagnosed as a functional problem by the psychiatric consultant, one had an eczema eventually diagnosed as neurodermatitis by the dermatologist, and one had bronchial asthma of moderate severity.

Four other patients developed nausea, vomiting, and/or diarrhea during the course of the trial period which necessitated interruption of the program. All four of these patients developed their symptoms on, or slightly before, the seventh day. In the records of nine other patients there was note of slight nausea and vomiting, the occasional refusal of a feeding, or mild diarrhea. The program was completed in all the latter instances.

A second general aspect of the situation to which attention had to be directed was the over all effect of these formulas upon the patients. Elimination and rotational dieting have been rightfully criticized by some because their

use frequently leads to inadequate intakes and depleted nutritional states. Since the trial period was a short one, after completion of which effort was made to restore an adequate, balanced intake to the patient it was not felt that the trial would precipitate deficiency states. Caloric intake was kept at 1,200 calories or above. In thirty eight instances where there was adequate and accurate record of body weight, twenty six patients lost an average of 42 pounds each, five patients gained an average of 21 pounds each and in seven instances there was no change in body weight. In no instance was there any clinically demonstrable detrimental effect upon the patients other than the gastrointestinal symptoms noted. These symptoms quickly subsided upon termination of the diet.

Considerable difficulty was experienced in evaluating the specific result of trial of diet upon allergic manifestations. Despite the fact that the plan appeared to lend itself to precision in this respect and to the development of objective criteria for making such decisions changes were frequently relative and subjective responses had to be taken into consideration. Responses to diet trial, however, could be grouped into the following four categories:

(1) Patients in whom there appeared to be no food factor. These patients completed ten days of diet without change in their symptoms.

(2) Instances in which there appeared to be a definite food factor. Symptoms of these patients cleared while on synthetic diet and were reproduced by additions to the formula.

(a) If additions were simple and flare resulted single allergens were identified.

(b) If groups of foods were added with resultant flare a definite food factor was considered present, even though a specific allergen was not identified.

(3) A small group of patients who experienced complete remission of symptoms upon the diet, but who subsequently tolerated return to general diet without reappearance of symptoms.

(4) Instances in which trial of diet was unsatisfactory for one of the following reasons:

(a) Failure to complete the course

(b) Uncontrolled collateral therapy

(c) Equivocal result

(d) Inadequate follow up contact with patient

Table III summarizes the results in fifty one patients who received synthetic diets.

In Group 1 foods were presumptively eliminated as a cause of symptoms. The fifteen patients in Group 2 had a definite food factor. It follows therefore that in thirty eight instances information of benefit to both patient and physician was obtained.

In the group of fifteen patients in whom there was thought to be a definite food factor twenty three separate allergens were diagnosed. Milk was the most frequent offender (eleven times) wheat white potato and

TABLE III

DIAGNOSIS	GROUP 1	GROUP 2	GROUP 3	GROUP 4	TOTAL
Perennial rhinitis	1				1
Bronchial asthma	12	5	1	8	26
Pulmonary emphysema	2				2
Bronchiectasis	1				1
Atopic eczema		5		2	7
Chronic urticaria	2				2
Chronic ulcerative colitis	1	1	1		3
Allergic colitis		1			1
Cephalalgia	1			1	2
Erythema multiforme	1				1
Vascular allergy		1			1
Chronic conjunctivitis	1				1
Chronic ulcerative stomatitis	1				1
Lupus erythematosus		1			1
Periarteritis nodosa		1			1
Total	23	15	2	11	51

beef were next (each three times). Not all patients in this group had been skin tested, but the majority had. For fifteen of the twenty three allergens demonstrated, there were both scratch and intracutaneous tests for comparison. In but four of the fifteen instances were there positive tests, either scratch (one) or intracutaneous (three), of *any* degree of positivity. This lack of correlation between skin testing and clinical observation in food allergy is in accord with general opinion as to the diagnostic specificity of skin testing for food allergens.<sup>14</sup>

#### DISCUSSION

Several considerations relative to the foregoing should be discussed in somewhat greater detail. First, the nonallergenicity of the formulas used cannot be assumed. The possibility remains that milk-, fish-, pork, mutton, or corn sensitive patients might continue to have allergic symptoms secondary to the ingestion of minute amounts of these substances remaining unaltered and undetectable in the final product. In view of the presence of short chain polypeptides, there remains the potential danger of immunologic reaction.<sup>15</sup> An enzymatic casein digest will not sensitize laboratory animals,<sup>16, 18</sup> and clinical observations supporting the theoretic nonallergenicity of these preparations have been made. For instance symptoms of patients known to be milk sensitive have been observed to clear upon an enzymatic digest of casein, and later to flare with the addition of milk to the basic formula. The final answer to this question should be withheld pending further clinical investigation and trial.

It should be re-emphasized that unpalatability is the chief objection encountered in the use of these diets. Our results have not been tabulated for each of the formulas because practical differences between them with respect to acceptability or clinical result were not apparent. It seems doubtful that any patient with less than major allergic symptoms would elect to remain upon such a program for the full trial period. Because of this impression, the procedure almost uniformly was reserved for inpatients.

The importance of controlling environmental factors the dust allergen and concomitant infection prior to embarking upon a diagnostic dietary regime cannot be overemphasized. On several occasions it was observed that proper consideration of these factors obviated the necessity for dietary trial which had originally been contemplated. The presence of an active infectious process, at least in the respiratory tree contraindicates a trial of one of the synthetic formulas. For example one patient with bronchiectasis superimposed on an allergic asthma could not be controlled from the standpoint of the asthma by any means during a flare of the pulmonary infection. After control of the pneumonitis with antibiotics and postural drainage residual asthma cleared rapidly upon institution of a synthetic diet.

It again should be pointed out that patients for whom diet trial was ordered were those whose symptoms were more than moderately incapacitating. The total number forms but a small though unimpaired percentage of the cases seen in Allergy Clinic.

The cause of gastrointestinal symptoms secondary to the use of these formulas is somewhat obscure. Aspartic and glutamic acid are reported to cause nausea and vomiting in both man and laboratory animals<sup>1,2</sup> and casein hydrolysates contain both of these amino acids. This may explain the development of symptoms but delay in their appearance until the sixth or seventh day of dieting is less readily explained on this basis than its appearance was more immediate. One patient who developed nausea and vomiting with Amigen orally was maintained satisfactorily on intravenous Amigen and subsequently tolerated a second trial of the oral preparation for a ten day period without the development of gastrointestinal tract symptoms. Although this patient was sustained with the parenteral preparation when unable to tolerate Amigen orally it was not the general policy to follow this plan. The end did not appear to justify the means even in the face of considerable literature attesting to the safety and efficacy of parenteral amino acid administration.<sup>1,8,10,12,13,17,19,20,21,4</sup>

Finally it should be kept in mind that the trial of diet occasionally was used as a differential diagnostic procedure in syndromes of obscure etiology. The final diagnosis was in some cases a condition not commonly thought to be of allergic origin. These cases have been included irrespective of this consideration because they represented instances in which synthetic diets were utilized in differential diagnosis. The recorded results (Table III) usually reflected the accepted nonallergic etiology of the disorder (namely pulmonary emphysema bronchiectasis) but in two instances (lupus erythematosus periarthritis nodosa) there was apparent benefit from the formulas in disease entities not commonly held to be allergic in origin. The patient with lupus erythematosus was a middle aged white man who was the victim of a disseminated process affecting most of the major organ system. A skin biopsy was reported as compatible with lupus but a skin muscle biopsy from a different site revealed essentially normal tissues. The clinical impression at the time of discharge was probable lupus erythematosus and follow up contact with the patient was insufficient to verify or disprove the diagnosis. The

symptoms of this individual twice cleared upon synthetic diets and twice flared with additions. Periaenteritis nodosa occurred in a middle aged, white woman. The diagnosis was made at the time of necropsy. Prior to death the patient had had almost intractable asthma for many months. There was associated high grade (20 to 50 per cent) eosinophilia observed in peripheral blood as well as transient pulmonary consolidations which appeared serially in different locations in the lung parenchyma. These were clearly demonstrated by successive chest x-rays. In the last six months of life, the only time of relative freedom from asthma were those periods when the patient subsisted upon synthetic formulas only. The example of this latter patient may be suggestive evidence of an allergic etiology for periaenteritis nodosa, supporting Rich's recent observations<sup>30</sup>. The instance of probable lupus erythematosus can be regarded as nothing more than a recital of observations which at this time cannot be extended to any conclusions.

In comparison with previously reported use of synthetic diets the formulas discussed herein would appear to be potentially less allergenic than any of the others. A more varied and larger series of cases has been reviewed than previously.

#### CONCLUSIONS

A group of fifty-one patients suffering from various ailments thought to be entirely or in part, of allergic origin has been studied by means of synthetic diets. In thirty-eight of these patients, or 74 per cent, it was felt that the trial afforded information of definite positive benefit to both the patient and physician information which could not have been acquired by other methods in so short a time with equal clarity.

The method would seem to supply another worth-while, safe, and sound approach to the problem of exact diagnosis of food allergy, although at the moment, in its present form, it does not appear practicable for outpatient use or for use upon patients with less severe allergic manifestations.

Until more clinical experience and study have justified the assumption that these formulas are nonallergenic, positive diagnoses of food allergy may be more valid than the negation of its presence after trial of synthetic diet.

The authors wish to acknowledge their appreciation of the work of Miss Ann Renner, Therapeutic Dietician, University Hospital in the planning, preparation, and supervision of the serving of the synthetic formulas discussed herein.

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## THE LIFE SPAN OF THE MEGALOCYTE AND THE HEMOLYTIC SYNDROME OF PERNICIOUS ANEMIA

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THERE is now general agreement that pernicious anemia is a deficiency disease. The ability of the liver extract principle to change the megaloblastic maturation arrest to a normoblastic type of red cell production seems to demonstrate that the site of the primary action of the antipernicious factor is located in the marrow.

Patients with Addisonian anemia also show regularly a severe disturbance of the pigment metabolism. There is usually mild retention jaundice. The hemolytic index,<sup>1</sup> that is, the urobilinogen excretion in the feces correlated with the total mass of circulating hemoglobin, is far in excess of normal values.<sup>2</sup>

These abnormalities of the pigment metabolism apparently are indicative of an existing hemolytic process since they also are observed in all other known hemolytic syndromes. However, contrary to these other types of hemolytic disorders, untreated cases of pernicious anemia show no increase or only a very slight increase of the reticulocytes in the circulating blood.<sup>3</sup> If reticulocytosis is evaluated as a measurement of red cell replacement and the high pigment output as an indication of the simultaneously existing increased erythrocyte disintegration, a considerable imbalance in favor of erythrocyte destruction would be present in untreated pernicious anemia. Consequently the peripheral blood should become rapidly depleted of erythrocytes, resulting in an early death of the patient. Clinical observations are not in agreement with such a mechanism. Therefore Addisonian anemia often has been considered not to be a true hemolytic syndrome. In order to account for the excessive bile pigment output, various explanations have been suggested. It has been postulated either that hemoglobin is destroyed in the marrow without ever entering the circulation,<sup>4</sup> or that the excessive urobilinogen is not derived from hemoglobin at all but represents rather pigment formed from other substances.<sup>5</sup> All these hypotheses tacitly imply that the morphologically abnormal megalocytes, after having reached the circulation, have then a normal survival time.<sup>3</sup>

In previous publications<sup>6, 7</sup> it has been pointed out that the common denominator of all hemolytic syndromes regardless of the great variety of causative mechanisms is to be found in a shortened life span of the various types of cells involved. In order to determine whether Addisonian anemia may be classified as a true hemolytic syndrome, estimations of the survival time of erythrocytes from patients with untreated pernicious anemia were performed in this study.

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## MATERIAL AND METHODS

The diagnosis of pernicious anemia in patients used in this investigation was based on a compatible clinical picture with histamine refractory achlorhydria, the presence of a macrocytic hyperchromic anemia, and a marrow examination showing the megaloblastic type of erythrocyte maturation accompanied by giant metamyelocytes. A moderate hyperbilirubinemia was found in the untreated patients. Furthermore, all patients responded well to liver treatment.

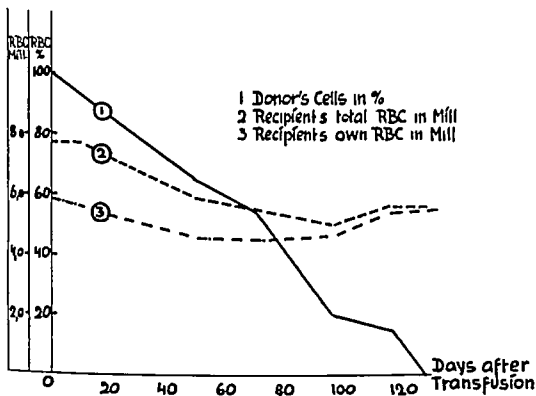


Fig. 1—Survival time of normal red cells in artificial erythrocytosis expressed in per cent of surviving cells

The methods used in this study have been described in detail in a previous publication. Determinations of the survival time were performed with the method of differential agglutination (A bby technique). Only young normal children (2 to 4 years old) were elected as recipients. Because of their small circulating blood volume a correspondingly small transfusion was found sufficient to result in a satisfactorily high addition of the donor's cells per cubic millimeter. Packed cells derived from 250 to 500 cc of whole blood were introduced, yielding an initial increase of 340 000 to 820 000 red blood cells in the recipient's total erythrocyte level. The initial values were established twenty four to forty eight hours after transfusion in order to avoid any significant inaccuracy due to an augmentation of the circulating fluid volume although it is realized that some of the transfused cells already may have been eliminated within the first twenty four to forty eight hours. After determining the starting point follow up studies were done twice weekly during the first month and from then on once every week.

Some of the recipients showed values of 5.5 to 5.8 million red cells following transfusion. In order to ascertain whether such an abnormally high count may have an influence on the survival time of the tagged cells, normal erythrocytes were transfused into children to such an extent that the total red count was brought up to more than seven million per cubic millimeter. Such an artificial erythrocytosis has no influence on the survival time. Fig. 1 shows an experiment in which the red count had been increased from an initial level of 5.8 to 7.7 million per cubic millimeter. After all transfused cells had disappeared the patient's red count was 5.5 million per cubic millimeter. The elimination curve of the foreign cell was a normal one and their average life span was 127 days, an entirely normal value.

## RESULTS

Nowadays the scarcity of untreated cases of pernicious anemia presents a major obstacle to investigations on the pathogenic mechanisms operating in this disorder. Our study was performed on four cases only\*. The hematologic data of the donors and also of the healthy recipients are compiled in Table I

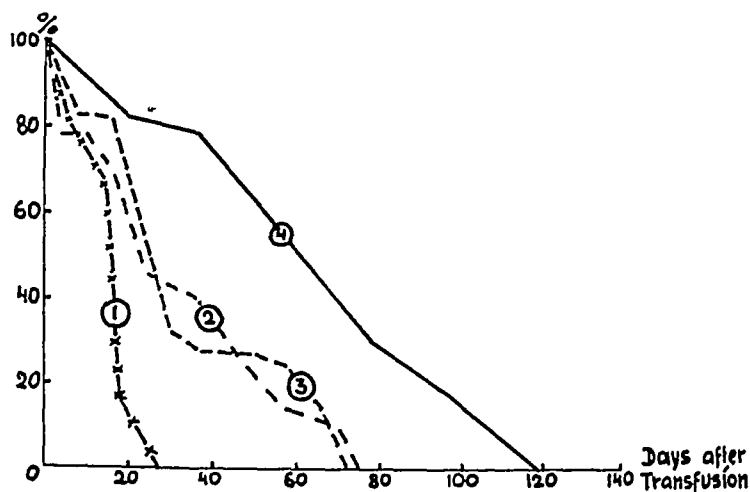


Fig. 2—Survival time of pernicious anemia cells in normal environment expressed in per cent of surviving cells

Fig. 2 shows the curves illustrating the disappearance of the donor's cells. The percentages of the surviving cells are plotted against the number of days following transfusion. Patients 1 and 2 were patients with pernicious anemia in relapse who had received no specific treatment for several months prior to the experiment. Their red cells were completely eliminated within twenty-eight and seventy-five days respectively when transfused into a normal environment. The profiles of their elimination curves are definitely abnormal and are similar to those seen in the other hemolytic syndromes caused by an intracorporeal abnormality of the red cells<sup>7</sup>.

Patient 3 had been under continuous treatment with liver for thirteen years. Then treatment was stopped entirely for about three years. A severe degree

TABLE I TRANSFUSION OF PE

PATIENT	AGE (YR)	SEX	DONORS		RBC (MILL)	RETCS (%)	TYPING FORMULA	AMOUNT USED TRANSF (CC)
			GM	%				
1	50	F	84	54	240	11	O Rh+	250
2	56	F	87	56	215	13	O Rh+	250
3	54	M	80	51	185	05	O Rh+	250
4*	69	F	157	101	560	08	O Rh+	250

\*Normalized blood picture after extensive liver treatment

\*We are indebted to Dr. S. Portis, Dr. L. Rappolt and Dr. S. O. Schwartz for permission to use their patients in this investigation.

of anemia developed and the patient was advised to enter the hospital. A few days prior to admission he took some liver pills. On the day of admission hemoglobin was 6.8 Gm (44 per cent) red count 1.66 million and reticulocyte count 3.8 per cent. The reticulocytes increased to 9 per cent on the third hospital day and then came back to values below 1 per cent on the tenth day. There was also a slight rise of the hemoglobin and red cell count to 5.7 Gm (56 per cent) and 2.11 million respectively. At the time blood was taken for the determination of the survival time, the hemoglobin had decreased to 5.0 Gm (51 per cent) and the red count to 1.85 million. Since the antipernicious anemia principle changes the abnormal megaloblastic maturation back to a normal development of the red cells, one may assume that two different populations of erythrocytes existed in the blood of this patient at the time of the transfusion, namely red cells manufactured with the participation of the liver principle and also typical megalocytes produced in the absence of the erythropoietic factor. As can be seen from Curve 3 (Fig. 2) visualizing the elimination of the foreign cells, 68 per cent of the transfused erythrocytes disappeared within thirty days, the remaining 32 per cent were completely eliminated after seventy-two days. The profile of the curve showing a distinct biphasic character is also in agreement with the assumption of the presence of two different populations of erythrocytes in the blood of this insufficiently treated patient. The suboptimal doses of antipernicious principle may also account for the total shortened survival time observed in this particular case.

Patient 4 had a normalized blood picture because of continuous liver treatment for many years. The average survival time was 119 days, an entirely normal value. This is in agreement with the recent findings of Mollison\* who also demonstrated a normal survival pattern of red cells obtained from adequately treated patients with pernicious anemia.

Our results, therefore, demonstrate that in untreated cases of pernicious anemia there is a shortened survival time and an abnormal elimination pattern which becomes normalized after adequate administration of liver extract.

#### DISCUSSION

Determinations of the average life span of normal erythrocytes transfused into patients with pernicious anemia were performed by Ashby<sup>9</sup> and also by

CELLS INTO NORMAL RECIPIENTS

SEX	RECIPIENTS				RESULTS	
	Hb		RBC BEFORE TRANSFUSION (MILL)	RETCS (%)	TYPING FORMULA	SURVIVAL TIME (DAYS)
	Gm	%				
F	13.7	88	4.55	0.9	A Rh+	71
M	13.1	84	4.96	0.4	B Rh+	75
F	14.5	93	5.39	0.1	A Rh+	72
F	11.9	77	4.12	0.8	A Rh+	119

Wearn and co-workers<sup>10</sup> as far back as 1921. A normal survival time of these transfused erythrocytes was found to be present. Recently Mollison<sup>8</sup> has confirmed these observations by means of the most refined modern techniques. Therefore an extracorporeal mechanism damaging all circulating erythrocytes at random and thus influencing the life span is not demonstrable in pernicious anemia.

The survival time of the megalocytes (pernicious anemia erythrocytes) was determined with the Ashby technique by Wearn and associates (in 1922) who transfused these abnormal red cells into another patient with pernicious anemia. No abnormal behavior of the transfused corpuscles was found in this single instance. Morawitz<sup>11</sup> transfused normal red cells into a patient with severe pernicious anemia to such an extent that the majority of all the erythrocytes in the patient's circulation belonged to the donor's group. Since the anemia improved for a longer period of time but no change occurred in the greatly increased pigment output in the feces, Morawitz concluded that the transfused normal erythrocytes survived much longer than the pathologic megalocytes.

In 1945 one of us<sup>6</sup> classified pernicious anemia as a true hemolytic syndrome caused by the presence of an intracorporeal anomaly of the erythrocyte. It was emphasized that hemolytic syndromes are not always produced by an erytholytic activity (immune bodies, hypersplenism) but that structurally defective erythrocytes, when exposed to the normal means of destruction, disintegrate much more rapidly than normal red cells. By using the method of cross de-termination of the survival time of the red cells,<sup>6, 7</sup> it is possible to distinguish in any given case whether an extra- or an intracorporeal mechanism is involved. When normal erythrocytes, transfused into a recipient with a hemolytic syndrome, survive normally, whereas the patient's own red cells, transfused into a normal person, have a considerably shortened life span, an intracorporeal abnormality may be suspected. Contrariwise, when normal red cells, transfused into the patient with the hemolytic syndrome, are as rapidly destroyed as the patient's own cells, the presence of an extracorporeal mechanism may be assumed.

While this study was in progress, we came across a report of Loutit<sup>1</sup> to the Royal Society of Medicine stating that he had transfused erythrocytes from two patients with untreated pernicious anemia into normal recipients and had observed a survival time of thirty and sixty days respectively. This is quite in accordance with our observations reported in the present paper. Although the elimination curves of Loutit's and our cases are all definitely abnormal, there is a considerably wide range of the average life span which is probably explained by the individual variability of the available antipericious principle for the manufacturing of the cells.

The demonstration of an abnormal life span of the megalocyte characterizes Addisonian anemia as a true hemolytic syndrome since the common denominator of all hemolytic disorders regardless of the great variety of causative mechanisms has been found to be a shortened life span of the various types of cells involved.

Although the shortened survival time undoubtedly accounts for an increased pigment production, the question arises whether it offers a truly satisfactory explanation for the excessively high output of bile pigment regularly observed in untreated cases of pernicious anemia.

The abnormalities of the pigment metabolism existing in this disorder have always been difficult to interpret. Watson<sup>3</sup> in his extensive review has critically discussed this problem. Whereas in the other hemolytic syndromes there is a high reticulocyte count indicating considerable red cell replacement untreated cases of pernicious anemia show no increase or only a very slight increase of the reticulocytes in the circulating blood. Whipple was the first to point out the great discrepancy between apparent blood regeneration and destruction in untreated pernicious anemia and he expressed his disbelief that the increased pigment excretion represents increased hemoglobin destruction. He postulated that the excessive urobilinogen is mostly derived not from the hemoglobin of the circulating red cells but rather from an increase of a hypothetical pigment complex.<sup>4</sup> This pigment complex was considered to originate from hemoglobin derivatives, food products and body proteins. Jedlicki<sup>4</sup> assumed that the elevated bile pigment output stems from hemoglobin only which however, is predominantly destroyed in the marrow. Both hypotheses have tacitly implied that the morphologically abnormal megalocytes after having reached the circulation have then a normal survival time.

Fig. 3 visualizes the hypothetical relationship between the interdependent factors involved in maintaining the erythrocyte level<sup>5</sup> (A) under physiologic conditions, (B) in the common hemolytic syndromes (for instance hemolytic jaundice, sickle cell anemia), and (C) in pernicious anemia according to the theories mentioned.

It should be emphasized however that Heilmeyer<sup>13</sup> and many other investigators (Lit. see Watson<sup>8</sup>) have rejected these hypotheses and maintained that the excessive urobilinogen output could be adequately explained on the basis of an increased hemoglobin destruction particularly if the extreme hyperplasia of the marrow which exists in pernicious anemia is taken into account.

At first glance the demonstration of a shortened survival time of the megalocytes seems to make any special hypotheses concerning the increased pigment output superfluous. However recent investigations<sup>14</sup> demonstrate that the whole problem is much more complicated. Shemin and Rittenberg<sup>15</sup> showed in 1946 that oral feeding of glycine labeled with N<sup>15</sup> results in the incorporation of N<sup>15</sup> in the heme molecule of the red blood cells. The labeled porphyrin remains in the erythrocytes until these cells disintegrate and is then transformed into stercobilinogen. By means of this technique the same figure for the average life span of normal erythrocytes was obtained as previously determined with the method of differential agglutination. However when labeled stercobilin was isolated from the feces and its amount correlated with the rate of erythrocyte disintegration it was found that a significant portion of the normal pigment production is apparently derived from sources other than hemoglobin.<sup>14</sup> In one case of untreated pernicious anemia a considerable increase of this extra

pigment was also demonstrable. Therefore these findings seem to support Whipple's hypothesis. Quite obviously an entire reinvestigation of the pigment metabolism in all the hemolytic syndromes now becomes necessary. However, the new evidence does not in any way reflect upon our interpretation of the significance of the shortened survival time of the megalocytes.

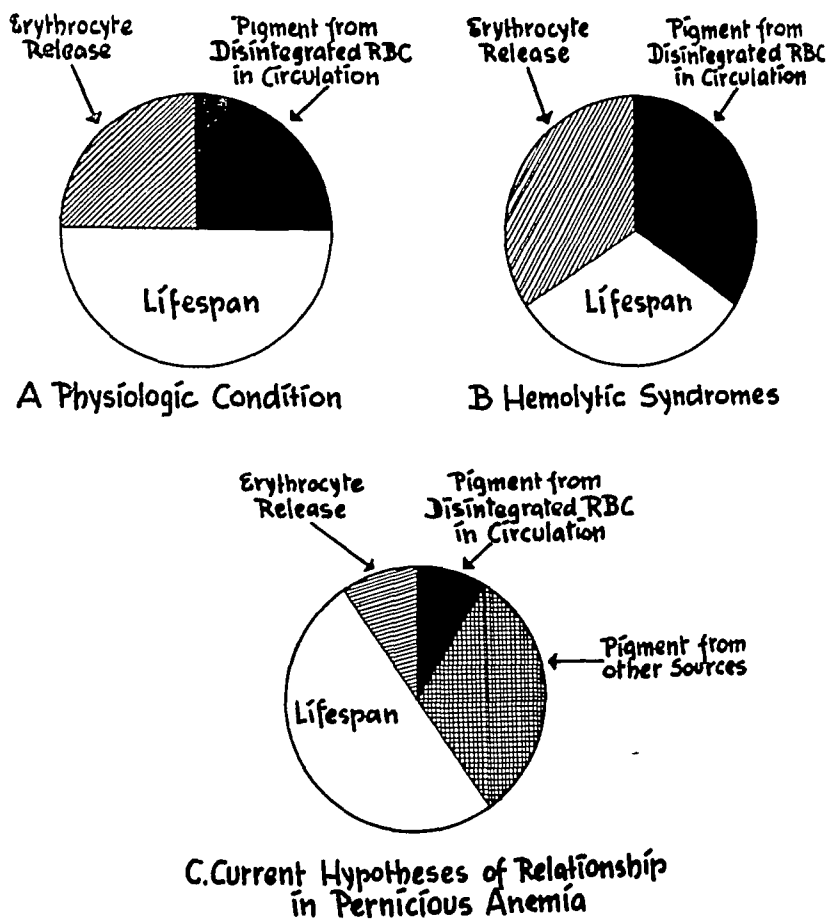


Fig 3—Relationship of the interdependent factors maintaining erythrocyte level

The absence of any considerably increased reticulocyte count in untreated pernicious anemia is of particular interest. It must be assumed that in this disorder replacement of erythrocytes occurs predominantly by means of non-reticulated megalocytes.<sup>16</sup> Apparently completely matured although qualitatively abnormal corpuscles are released into the circulation. This is quite a distinguishing feature since no such abnormality of replacement is demonstrable in the other types of hemolytic anemia. There has been some speculation that the liver extract principle may contain a specific reticulocytogenic factor. Oliva and Frascarelli<sup>17</sup> report that plasma obtained from patients with a high reticulocyte count caused a definite increase in reticulocytes when injected into normal persons. This was observed with blood from patients with pernicious anemia.

at the reticulocyte crises following liver treatment and also in patients with microcytic hypochromic anemia treated with iron. Oliva and Frascarelli suggest that in pernicious anemia this hypothetic reticulocytogenic factor acts in combination with the maturation principle. Somewhat against this assumption is the satisfactory hematologic response of patients with pernicious anemia treated with pteroylglutamic acid.

The demonstration that pernicious anemia is a true hemolytic syndrome does in no way invalidate the concept of this disorder as a deficiency disease. It is because of the absence of the maturation principle that defective erythrocytes enter the circulation and are then eliminated more rapidly than normal ones. Thus pernicious anemia is another example of a hemolytic syndrome caused by an intracorpuseular mechanism, namely a poorly constructed cytoskeleton of the erythrocytes.

#### SUMMARY

The red cells of patients with untreated pernicious anemia have a shortened survival time. After adequate treatment the life span of the erythrocyte becomes normal.

The demonstration of a shortened survival time of the megalocyte permits the classification of pernicious anemia as a true hemolytic syndrome since the common denominator of all hemolytic anemias regardless of the great variety of causative mechanisms has been found to be a shortened life span of the various types of cells involved.

The significance of the finding of a shortened survival time for the explanation of the abnormal pigment metabolism in pernicious anemia is discussed.

Quite different from the other types of hemolytic anemia replacement of red cells seems to occur by means of nonreticulated erythrocytes.

The demonstration that pernicious anemia is a true hemolytic syndrome does in no way invalidate the concept that this disorder is a deficiency disease. It is because of the absence of the maturation principle that defective red cells enter the circulation and are then eliminated more rapidly than normal ones. Thus pernicious anemia is an example of a hemolytic syndrome caused by an intracorpuseular mechanism, namely a poorly constructed cytoskeleton of the erythrocytes.

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# THROMBOPENIC PURPURA THE FAILURE OF DIRECT BLOOD TRANSFUSION TO RAISE THE PLATELET LEVEL\*

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WILLIAM S ADAMS M D  
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IT IS the purpose of this report to show that the platelet level of patients with thrombopenia cannot be elevated significantly for any period of time by means of massive direct transfusions of blood. For a long time it has been our feeling as well as that of others, that blood transfusions do little in patients with thrombopenic purpura except to replace blood which has been lost by bleeding. However following blood transfusion in the amounts ordinarily used the dilution factor is so great that it has been impossible to determine accurately whether platelets in the transfused blood remain for any appreciable time in the circulation of the recipient.

Recently it was demonstrated in this laboratory that the circulating platelet level of the cat can be substantially raised for a period of a few days following cross circulation by way of carotid to carotid anastomoses with a normal animal.<sup>1</sup> The technique in effect, constituted a method of giving the thrombopenic recipient a massive direct blood transfusion through a continuous endothelial anastomosis. The experimental results made it seem pertinent to determine whether the platelet level in human subjects with very low platelet counts could be raised by massive direct transfusions of whole blood. On a priori grounds it seemed that massive direct transfusions should appreciably raise the platelet level and that if the blood platelet in man has a normal rate of utilization of the same general order of magnitude as that found in the cat this increased value should be demonstrable for a period as long as a few days. It was argued further that such an increase in the platelet level should have a favorable effect on hemostasis in thrombopenic individuals, and thus massive transfusions of whole blood would offer more benefit than the conventional indirect transfusion of 1 or 2 units of blood. Since the animal experiments had shown the life span of blood platelets to be much greater than that of the leucocytes under comparable conditions, it was hoped that thrombopenic purpura could be combatted by transfusions more effectively than agranulocytosis. Accordingly massive direct blood transfusions were given to two patients with marked thrombopenia.

The medical literature records only two pertinent publications of which we are aware. Duke<sup>2</sup> in 1910 reported that direct blood transfusion both raised the platelet counts and improved the hemostasis of three patients with thrombo-

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penic purpura In one case a rise in platelet count of 120,000 per cubic millimeter was noted No data are given as to the method of direct transfusion employed or as to the amount of blood transfused Kiasso<sup>2</sup> in 1927 likewise recorded beneficial effects from blood transfusion in thrombopenic purpura In one case, following the transfusion of a single unit of blood, the platelet count rose from 11,000 to 114,000 per cubic millimeter one day following transfusion In the second case no significant elevation in platelet level occurred until six days after the transfusion of 450 cc of blood The amounts of blood transfused and the time intervals concerned are such that it seems unlikely that survival of transfused platelets could per se account for the observed elevations in the platelet counts

The failure of blood transfusion to replenish satisfactorily the circulating nonerythrocytic formed elements when these are dangerously low can be attributed to at least five possible causes (1) The life span of these elements may be so short that when transfused they rapidly disappear merely in the course of normal utilization This is true in the case of the white blood cells (2) The elements may be qualitatively or quantitatively altered within a short period in stored blood This factor is of undoubted importance (3) The transfused elements are rapidly dispersed in the comparatively large blood volume of the recipient and, in many instances, are diluted to the level of insignificance on this basis alone This is certainly true in instances where only 500 cc or less of blood are transfused (4) The abnormal state which prompted the transfusion initially may result in an abnormally rapid rate of disappearance of the transfused elements It is, of course, difficult to exclude this situation (5) The recipient may destroy the donor elements more rapidly than his own No satisfactory way of proving this exists

CASE 1—J J, a 37 year old Italian man, was first admitted to Strong Memorial Hospital in 1943 with complaints of dizziness, blurred vision, numbness and tingling of the left hand and left leg, and difficulty in walking There was generalized pallor of the right optic disc No objective sensory changes were observed There was some ataxia in the finger to nose test with the left hand The blood Wassermann was negative The erythrocyte count was 5,820,000 per cubic millimeter, the hemoglobin was 15.8 Gm per cent, and the leucocyte count was 10,900 per cubic millimeter with a normal differential A diagnosis of multiple sclerosis was made and the patient was given a course of intravenous typhoid vaccine The patient was able to continue working and except for episodes of transient numbness the symptoms improved

In December 1945, the patient began to note pallor, exertional dyspnea, weakness, and fatigue He was admitted to the Rochester General Hospital where he was found to have a severe anemia Sternal marrow aspiration specimens suggested an aplastic bone marrow The patient received numerous blood transfusions with but transient benefit and was referred to Strong Memorial Hospital on April 2, 1946, for further study At the time of admission there were petechiae over most of the legs, and the patient's story indicated that a petechial rash had first appeared three months previously in the areas between the first and second fingers bilaterally The frequent occurrence of small hematomas had been noted for two months before admission There had been frequent free bleeding from the gums for two weeks prior to admission No history of ingestion of drugs of the type which are known to predispose to aplastic anemia could be elicited

On physical examination, with the exception of pallor of the optic discs the pertinent findings were limited to generalized pallor and the wide distribution of hemorrhagic mani-

festations. There were petechiae on the tongue and the mucous membrane of the mouth oozing from the gums, clotted blood in the nostrils, and diffusely scattered petechiae over most of the body which assumed the proportion of hemorrhagic rash on the lower extremities. The spleen was not palpable.

At the time of admission the red blood cell count was 1,860,000 per cubic millimeter, the hemoglobin 12 Gm. per cent, and the leucocyte count 3,050 per cubic millimeter with but 29 per cent granulocytes. The urine showed 3 plus albumin and there was a positive test for blood. The stool showed a 3 plus guaiac test for blood. The clotting time (Lee White method) was 9 minutes and clot retraction was poor, the clot being very friable and melastic. The bleeding time was in excess of 42 minutes. The reticulocyte count was 18 per cent. The platelets were virtually absent from the blood film. A specimen of sternal marrow was removed surgically and a pathologic diagnosis of aplastic anemia was made. Despite indirect blood transfusion and vitamin K, the clinical course was progressively downhill. Continued bleeding was a troublesome problem and it was decided to attempt massive direct blood transfusion.

On April 5 the patient's blood picture was as follows, prior to transfusion: Erythrocytes 1,500,000 per cubic millimeter, hemoglobin 14 Gm. per cent, volume of packed cells 130 per cent. The platelet counts on three separate determinations (Rees-Ecker method) were 1,000, 6,000, and 6,000 per cubic millimeter. The blood film was almost completely devoid of platelets. A direct transfusion of 690 cc. was given and the patient developed a pyrogenic reaction necessitating discontinuation of the transfusion. Three hours after the transfusion the platelet count was 0 per cubic millimeter on two determinations. The following morning the erythrocyte count was 2,090,000 per cubic millimeter, the hemoglobin 63 Gm. per cent, and the hematocrit, 17.0 per cent. The platelet count was 2,000 per cubic millimeter. In a two-hour period 1,500 cc. of whole blood were transfused by the multiple syringe method. Despite this massive direct transfusion from normal donors the platelet count two hours afterward was but 6,000 per cubic millimeter and there was no discernible elevation in numbers of platelets on careful examination of the blood film. Slow oozing of blood from the nose, present before the transfusion, never abated. The patient continued to bleed and died on April 13. No autopsy was performed.

CASE 2—C. C., a 53-year-old white man was admitted on Sept. 1, 1947, to the Eye Service complaining of a cataract of the right eye. The history indicated a hemorrhagic diathesis dating back to childhood with recurrent episodes of petechiae and easy bruising covering a period of many years. A splenectomy had been performed for idiopathic thrombocytopenic purpura in 1936. Following this there was only slight improvement in the bleeding tendency and the platelet count had remained depressed. However, in the year prior to admission there had been no significant episodes of bleeding.

The patient had noted failing vision since 1929 when he was found to have cataracts. In 1943 an iridencleisis had been performed following which the patient developed hemorrhagic glaucoma of the left eye. Since that time he had been virtually blind in the left eye, being able to discern only bright light and the vaguest outline of objects. Vision in the right eye was becoming progressively smoky and the patient was threatened with nearly complete blindness. Pertinent facts in the past history also included nephrolithiasis treated medically because of the bleeding tendency, hernia at the site of the previous splenectomy, and deafness of a mixed type in the right ear.

On physical examination the lens of the right eye was cloudy white and vision was greatly reduced. Vision was virtually absent in the left eye. There were surgical scars in the left upper quadrant of the abdomen and there was slight bulging in this area when the patient strained. There were a moderate number of scattered petechiae and a few larger purpuric areas. Physical examination was otherwise essentially normal.

The erythrocyte count was 5,700,000 per cubic millimeter, the hemoglobin, 18.5 Gm. per cent, and the leucocyte count 10,850 per cubic millimeter with a normal differential formula. The platelet counts were 26,000, 27,000, and 23,000 per cubic millimeter on three determinations. The platelets were greatly reduced on the blood smear. The bleeding time was 5½

minutes on one occasion and 11½ minutes on another. The clotting time was within normal limits and the clot retracted somewhat. The blood fibrinogen was 328 Gm per cent.

It was felt that removal of the cataract from the right eye was essential if the patient was to avoid blindness but that the hemorrhagic diathesis reduced materially the chances of successful operation. It was therefore decided to search for an accessory spleen prior to attempting to remove the cataract. In preparation for splenectomy the patient was twice phlebotomized and a total of 1,000 cc of blood withdrawn. He then received 1,000 cc of blood by direct transfusion from three normal donors. The Pennell apparatus was employed and by this method it was possible to transfuse 500 cc of blood in eight minutes, assuring that the formed elements in the blood were a very short time outside the body. Unfortunately, technical difficulties with the platelet counting dilution fluid did not permit quantitative evaluation of the attempt to increase the blood platelets in this case. However, careful examination of blood films for platelets were made. It was felt that a very minimal increase in the numbers of blood platelets transiently occurred, but the increase was far below expectations and had disappeared completely within a few hours. The patient was operated upon the following day. There were many bleeding points but the patient did not bleed as much as was anticipated. No accessory splenic tissue could be found.

The only recourse left was to attempt to remove the cataract of the right eye in spite of the risk of hemorrhage. The alternative was blindness for the patient. Therefore a second attempt to prepare the patient was made using the same direct transfusion technique. As before, after phlebotomy, the patient received via the Pennell apparatus 1,500 cc of blood from three normal donors within a three hour period. The day prior to transfusion the platelet counts were 18,000 and 23,000 per cubic millimeter and the bleeding time was 12 minutes. The morning of transfusion the platelet count on several determinations varied between 10,000 and 16,000 per cubic millimeter and the bleeding time from 10 to 17 minutes. Immediately after the transfusion the blood platelets had risen to between 50,000 and 80,000 per cubic millimeter, the bleeding time was 3 minutes. There appeared to be a moderate increase in the numbers of platelets on the stained films. However, two hours later the platelet count had fallen to 24,000 to 28,000 per cubic millimeter and the bleeding time was 5½ minutes. An hour later the platelets were below 20,000 per cubic millimeter. The morning following transfusion (which was the morning of the operation) the platelets were 20,000 per cubic millimeter and the bleeding time was 19 minutes. During the post operative period the eyeball filled with blood, and at the time of this report, six months after operation, the patient was almost completely blind.

#### DISCUSSION

In each of the three transfusions reported, a minimum of 1,500 cc of whole blood was administered within a short period of time. If the blood volumes of the recipients were somewhere within the normal range, no more than three to fourfold dilution of the transfused elements would be expected. If the blood platelets were received undamaged this would mean an elevation of at least 80,000 to 100,000 in the platelet count. On the basis of the experiments with animals, a detectable platelet increase persisting for an appreciable period of time would be anticipated. However, in only one of the three experiments reported was a significant increase noted in the recipient, and even in this case it was so ephemeral as to be of little practical value.

The reasons for the unsatisfactory results are not entirely clear. In Case 1 the patient (J J) had aplastic anemia, and, presumably, the fundamental defect to which the thrombopenia was due was merely a lack of platelet precursors. Though it cannot be said with certainty, there is no reason to believe that abnormally rapid platelet destruction was a factor in this case. Yet no elevation

in platelet count occurred and hemostasis as evidenced by slow oozing of blood from the nose remained unimproved. It was thought that the use of multiple glass syringes might have meant that few or no platelets were actually being reinjected into the recipient, but platelet counts on blood allowed to stand for periods of time up to five minutes in glass syringes indicated that large numbers of these elements were undoubtedly still present in the injected blood. However, it is quite possible that qualitative alterations in platelets during their brief stay outside the body may have resulted in their premature death on reinjection. Still another possibility is that the preceding thrombopenia created an abnormal need for platelets and that the injected platelets were almost immediately utilized for this reason. This seems unlikely. It is also possible that donor platelets are not utilized in a normal manner in human subjects or that their normal rate of utilization is far greater than that found in animal experiments.

In Case 2 an attempt was made to obviate the effects on the platelets of being outside the body by using an apparatus which would reduce this period outside. The Pennell apparatus consists essentially of a short rubber tubing running between donor and recipient the blood being pulled from donor to recipient by means of a rotating worm. As much as 500 cc of blood were transferred from donor to recipient within eight minutes so that no unit of blood could have remained more than a very brief period outside the vascular system. Despite this results were disappointing and the transient slight elevation in platelet count was of no practical clinical significance. This patient (C C), of course, had idiopathic thrombopenic purpura and it is not entirely clear whether or not platelet destruction takes place at a normal rate in this disease.

It should be emphasized that the direct transfusion techniques employed are by no means comparable to the continuous endothelial anastomosis of the animal experiments. Furthermore, in the animal work there was a much longer period of mixing of the blood of thrombopenic and normal animals and larger amounts of normal blood were mixed with thrombopenic blood. If comparable conditions had been present in the human subjects the results *might* have been different.

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# A SIMPLE AND RAPID METHOD FOR DEMONSTRATING SICKLING OF THE RED BLOOD CELLS THE USE OF REDUCING AGENTS

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## INTRODUCTION

THE practical importance of a rapid and simple method of detecting the sickling phenomenon is apparent to anyone familiar with the various clinical manifestations of sickle cell disease. Unrecognized, this condition may masquerade as a variety of clinical entities, including rheumatic fever, osteomyelitis, cerebrovascular accidents, and abdominal conditions such as acute cholecystitis or appendicitis, presumably requiring prompt surgical intervention. Sicklemia may also be the cause of otherwise unexplained mild or severe anemia. Thus the ready performance of a simple test for sickling of the red cells may be useful in the hospital, in the physician's office, or even under field conditions in which genetic or anthropologic studies are being undertaken.

The sickling of the abnormal red blood cells capable of this remarkable alteration of the normal discoidal form is closely correlated with the concentration of reduced hemoglobin. The sickling phenomenon appears when the oxygen tension in the gas phase with which the blood is in equilibrium is 40 to 45 mm of mercury or less.<sup>1,3</sup> Below this value the oxygen tension which causes manifest sickling depends on whether the active disease or only the so called trait is present.<sup>4</sup> A fall in pH within the physiologic range increases the tendency to sickling at a given oxygen tension because of the resulting greater percentage of reduced hemoglobin in the red cells. When the red cells in a sample of blood are sickled, they form an interlacing network of crescentic, filamentous structures and as a result do not separate readily from the plasma under the influence of gravity or even of the centrifuge. Consequently when a sample of blood is sickled its sedimentation rate is decreased. Due entirely to the sickling of the red cells the viscosity<sup>5</sup> of the blood sample is also increased, as is the mechanical fragility<sup>5,6</sup> of the red cells. These physical phenomena probably explain the thrombotic and hemolytic tendencies respectively which are so common in the clinical and pathologic manifestations of the disease.<sup>3,5</sup>

With the possible exception of the method recently proposed by Neuda and Rosen<sup>9</sup> which appears to depend on enzymic injury to the red cell surface, all clinical tests for sickling require the production of reduced hemoglobin. The resulting sickling is then judged by direct microscopic observation except when detected by the inhibitory effect of sickling on the sedimentation rate<sup>10</sup> in the

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tedious method of Winsor and Burch<sup>11</sup> Once sickled the red cells may be fixed in the sickled form with formalin solution<sup>4</sup> Removal of oxygen from the red blood cells is effected either by physical means such as exposure of the blood to low barometric pressure (vacuum pump)<sup>4</sup> or at normal barometric pressure by displacement of the oxygen by equilibration of the blood sample with gases such as hydrogen, nitrous oxide, carbon dioxide or nitrogen<sup>1,3</sup> Oxygen and carbon monoxide cause prompt reversion to the discoidal form A second principle employed is the reduction of the oxygen tension in the blood by denying access of more oxygen to the blood while that in the sample is being consumed metabolically This may be carried out *in vivo* by causing stagnation of the circulation in a finger<sup>2</sup> for a few minutes with a tourniquet before withdrawal of a drop of capillary blood into formalin solution<sup>4</sup> or for rapid coverage beneath a coverslip *In vitro* the same result may be achieved by allowing the consumption of the oxygen chiefly by the white cells of the blood sample to proceed in a sealed coverslip preparation<sup>12</sup> The utilization of oxygen in such a system may be accelerated by incubation at body temperature or by the addition of leucocytes<sup>4</sup> or of aerobic bacteria<sup>4, 13</sup> These methods are effective but do have the disadvantage of requiring special equipment such as vacuum pump gas equilibration chamber oxygen free gases stool filtrates bacterial cultures or incubator

#### METHOD

The simple method described here depends upon another principle namely the reduction of the hemoglobin of the red cells by a chemical agent Besides a drop of blood (which may be capillary blood taken directly from the ear or finger tip or venous blood defibrinated or oxalated) only a microscope microscopic slides and cover slips and the reducing agent are required Its only disadvantage is that because of the rapidity of its action it fails to distinguish the different susceptibilities to sickling of the red cells of active sickle cell disease and of the sickle cell trait After trying several substances ascorbic acid in the form of Cevalin was chosen It is readily available in the form of a buffered 10 per cent solution of the sodium salt with a pH of about 6.5 in sealed glass ampules containing 5 cubic centimeters Because this particular solution when diluted with water to a concentration of 2.06 per cent was found to be approximately isotonic for red cells, a small amount of a 2 per cent solution is prepared preferably from a freshly opened ampule by fivefold dilution with water immediately before use

The test has been in routine use in the Thorndike Memorial Laboratory of the Boston City Hospital for nearly two years and during this time has been taught to all Harvard second year medical students The performance of the test is as follows A small drop of the blood to be tested is placed upon a glass microscope slide and mixed with 1 or 2 drops of the diluted ascorbic acid solution The mixture is at once covered with a glass cover slip on which pressure is momentarily exerted in order to extrude excess blood and produce a blood film sufficiently thin to permit satisfactory examination of individual red cells using the high power dry objective of the microscope The preparation is inspected for the presence of sickling at fifteen minute or other convenient intervals while standing at room temperature

In originally determining the efficacy of the method the cover slips were sealed at the edges with hot paraffin for additional protection against the entrance of oxygen Control preparations were set up in a similar fashion except that either a drop of blood

Kindly supplied by Mr George B Walden of Eli Lilly & Company Indianapolis In 1940 according to Mr Walden the composition of 100 c.c. of the solution covered by a patent is used is as follows ascorbic acid 11.0 Gm sodium carbonate anhydrous powder 3.5 Gm sodium metabisulfite 0.5 gram

alone or a drop of blood mixed with 1 or 2 drops of 0.85 per cent sodium chloride solution was sealed beneath the cover slip. In practice, only the preparation containing the ascorbic acid solution need be made, and the sealing of the cover slip with paraffin is unnecessary.

### RESULTS

In Table I are shown the results of eighty tests for sickling made at various times on the blood of fourteen patients with sickle anemia, including both anemic and nonanemic individuals. In 75 per cent of the samples, definite sickling appeared within one hour when the 2 per cent ascorbic acid solution was used as described. Sickling was present in all by the end of 3.5 hours at room temperature. On the contrary, in none of fifty control samples of blood from the same patients to which either nothing had been added or, in most instances, 2 drops of 0.85 per cent sodium chloride had been added did sickling appear within an hour. Indeed, sickling was present in only about 13 per cent of the control samples at the end of three hours, and after twenty hours 11 per cent of the control samples still failed to exhibit sickling.

TABLE I EFFECT OF A BUFFERED 2 PER CENT SOLUTION OF ASCORBIC ACID CONTAINING 0.11 PER CENT SODIUM BISULFITE ON THE RATE OF FORMATION OF SICKLED CELLS IN SEALED WET COVER SLIP PREPARATIONS OF THE BLOOD OF FOURTEEN PATIENTS WITH SICKLE ANEMIA

INTERVAL REQUIRED FOR DEFINITE SICKLING (HR.)	EIGHTY EXPERIMENTS		CONTROLS		
	POSITIVE TESTS		NUMBER OF CONTROLS	POSITIVE TESTS	
	NUMBER	PERCENTAGE		NUMBER	PERCENTAGE
0.25	9	11.3	52	0	0
0.5	27	22.5	51	0	0
0.75	36	43.8	50	0	0
1.0	60	75.0	50	0	0
1.5	66	82.5	13	2	4.4
2.0	77	96.2	37	2	5.4
2.5	78	97.5	32	2	6.3
3.0	79	98	30	4	13.3
3.5	80	100	28	4	14.3
5.0			24	7	29.2
10			15	11	73.3
20			46	41	89.1

The figures given are summations of the number or percentage of preparations exhibiting 5 to 20 per cent sickling at the interval stated. The time for the performance of the observations on the controls at five and ten hours occurred in many instances during the night and as a result an inspection was not made. Once the preparation containing ascorbic acid had become sickled inspection of the controls tended to become less frequent. These factors led to the reduction in the number of controls observed in the interval between 1.5 and 20 hours as shown in the table.

### DISCUSSION

That the effect of the ascorbic acid solution in causing sickling was in fact due to the reduction of the hemoglobin and not to some other action was proved by its inability to produce sickling in a hanging drop preparation, that is, one freely exposed to air. The reducing action of the ascorbic acid solution, however, for the purposes of the test described here, was shown to be independent of the rate of metabolic consumption of oxygen by the leucocytes in the preparation. Thus, high and low white blood cell counts respectively were artificially produced in different portions of a single sample of heparinized venous blood.



from a patient with sickle cell anemia. By centrifugalization of the original sample which contained 7,200 white cells per cubic millimeter, removal of the buffy coat and its addition to another portion of the sample two portions were obtained which contained respectively 4,000 and 38,300 white cells per cubic millimeter. As was to be expected from the results of others<sup>4</sup> in similar experiments, sickling appeared in a sealed preparation of the blood with the high white blood cell count within forty five minutes, but failed to appear in similar preparations of the original blood and of the blood with the low white blood cell count even after fifteen hours. On the other hand, when to a drop of each of the three samples of blood was added a drop of the 2 per cent ascorbic acid solution in a sealed cover slip preparation, sickling appeared within one hour in the blood with the low white blood cell count and was observed to appear only a few minutes earlier in the other two specimens.

That besides the times saved in its performance the use of the ascorbic acid solution enhances the accuracy of the sickling test is suggested by the fact that about 10 per cent of the controls, as shown in Table I still failed to show sickling after twenty hours at room temperature. That acceleration of the process of sickling, which usually results from the incubation of the specimen may not avoid this difficulty is suggested by the recent observations of Shen, Flemin<sup>10</sup>, and Castle<sup>14</sup> who found that the red cells of patients with sickle cell disease may fail to sickle if the blood has been incubated for twenty four hours at 37.5 C in the presence of oxygen. Thus in actual practice it appears possible that if sickling in the sealed preparation in the usual clinical test is sufficiently delayed, the red cells of the specimen may lose more or less completely the power to become sickled. Possibly an unusually slow consumption of oxygen in specimens of blood containing relatively few leucocytes may be responsible for the negative results among the control specimens. At any rate occasionally it has been our experience in the past that sickling has failed to appear in sealed cover slip preparations even when they were kept in the incubator for twenty four hours, whereas sickling was readily demonstrable in the fresh blood upon exposure to carbon dioxide gas.

Reducing agents such as methylene blue, potassium ferrocyanide thioglycolate, and lactic and succinic acids were not as efficient as the buffered 2 per cent solution of ascorbic acid for the performance of the sickling test. That other reducing substances may be employed successfully was suggested to us at the completion of these studies by the report of da Silva<sup>1</sup> who states that a 2 per cent aqueous solution of sodium hydrosulfite caused rapid sickling in unsealed cover slip preparations of sickle cell blood. We found this particular compound,  $\text{Na}_2\text{S}_2\text{O}_4$ , not very active. Sodium thiosulfate  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  in 2 per cent solution caused no sickling at the end of fifteen hours. Sodium bisulfite  $\text{NaHSO}_3$ , however, in 2 per cent solution in water caused sickling of the same blood in fifteen or thirty minutes.

Sodium bisulfite (metabisulfite), which is present in a concentration of 0.55 per cent as a stabilizer of the sodium ascorbate in Cevalin, undoubtedly adds to the reducing power of that solution. However, a 2 per cent solution of ascorbic acid brought to pH 6.5 with sodium carbonate and without added bisulfite produced sickling within an hour. A 0.11 per cent solution of sodium bisulfite in 0.85 per cent sodium chloride, which is the concentration of bisulfite in the five fold dilution of the Cevalin, appeared to be about as active as the diluted Cevalin itself. The 2 per cent solution of sodium bisulfite was without question the most active of the substances tested. (See Addendum.)

The probability that solutions of any reducing agent may deteriorate on standing exposed to air suggests the desirability of using solutions freshly prepared from the salt. This is easily done with sodium bisulfite, but crystalline ascorbic acid proved to be entirely too acid and consequently injurious to the red cells without the addition of alkali. Even after opening the ampule, however, Cevalin appears to keep its reducing properties in the icebox for some days although its exact rate of deterioration has not been ascertained. Although not as rapid in its action as the 2 per cent aqueous solution of sodium bisulfite, it has the practical advantage of requiring only simple dilution rather than weighing in its preparation.

Because formalin is a reducing agent it was assumed that for this reason tissues from patients with sickle cell anemia when fixed with this substance more clearly exhibit sickling of the erythrocytes than do tissues fixed with Zenker's fluid.<sup>5</sup> This property of formalin is, however, not the explanation since experiments in which either previously sickled or unsickled red cells were mixed anaerobically with 10 per cent formalin in 0.85 per cent sodium chloride solution showed that the formalin fixed the red cells in whichever phase they were exposed to it. It should be pointed out, however, that the presence of sickled red cells in the capillaries of formalin-fixed tissues, though valid evidence of the sickle cell trait, does not necessarily demonstrate to what extent sickling was present during life. Sufficient anoxia to cause extensive erythrocyte sickling may have occurred only as an agonal event or only after the circulation had ceased with death or at the time of the surgical removal of the tissue. In contrast to formalin Zenker's fluid without added acetic acid when diluted to 10 per cent with 0.85 per cent solution of sodium chloride readily caused reversion of sickled red cells to more or less normal appearing discoidal forms. It is therefore probably because of this effect that the tissues of patients with sickle cell anemia when fixed with Zenker's fluid may not show the characteristically shaped red cells.

#### SUMMARY

A simple and rapid method of producing sickling of the red blood cells in wet cover slip preparations of the blood of patients with sickle cell anemia is described. The principle on which the test is based is the production of reduced hemoglobin in the red cells by the addition of a reducing agent. In order to perform the test, a drop of a fivefold aqueous dilution of Cevalin (approximating a 2 per cent solution of buffered ascorbic acid and also containing 0.11 per cent

sodium bisulfite) or a drop of 2 per cent sodium bisulfite  $\text{Na}_2\text{SO}_3$ , is added to a small drop of the patient's blood on a glass microscope slide. After mixing, a cover slip is dropped on the preparation and excess blood is expressed by gentle pressure in order to produce a film of blood sufficiently thin to permit inspection of individual red cells under the high power dry objective of the microscope. With the diluted Cevalin solution, sickling of the blood usually appeared within an hour and with the 2 per cent bisulfite solution it was often present within fifteen minutes at room temperature.

Appropriate experiments demonstrated that the rate of sickling of the red blood cells caused by the ascorbic acid solution is for the practical purposes of this test, unaffected by the rate of metabolic consumption of the oxygen by the white blood cells of the specimen. It was shown that formalin although a reducing agent, promptly fixes the red blood cells in whichever form they are exposed to it, whether sickled or unsickled. Zenker's fluid however caused already sickled erythrocytes to reassume a discoidal appearance which probably accounts for the fact that histologic preparations of tissues from patients with sickle cell anemia when fixed with Zenker's fluid may not exhibit sickled red cells in the capillaries as clearly as when the tissues are fixed with formalin.

#### ADDENDUM

Since this article went to press two communications have been reported. Dr Janet Watson, of the Long Island College of Medicine has reported by personal communication observations made by Dr James McGovern of Bellevue Hospital New York. These investigators observed sickling in cover slip preparations occurring in less than five minutes when using a 1 per cent solution of sodium acid sulfite (bisulfite),  $\text{NaHSO}_3$  Baker. The first appearance of any sickled cells around the periphery of the cover slip preparations was considered as the end point.

Dr Louis Thomas and Dr Chandler A. Stetson Jr have recently made a preliminary report on "Sulfhydryl Compounds and the Sickling Phenomenon." These authors report sickling in less than one half hour in wet preparations made with reducing substances such as a saturated solution of hydrogen sulfide (cysteine) (0.5 molar solution) and BAL (0.1 molar solution of 2,3 dimercaptopropanol).

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## ANTI A AND ANTI B ISOAGGLUTININ TITERS IN Rh IMMUNIZED PREGNANT WOMEN

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THE discovery of the ABO blood types, their heredity and antigenic properties and their significance in blood transfusions brought forth many years ago the question whether ABO incompatibility during pregnancy might be responsible for disease during pregnancy or in the newborn infant. Very few convincing cases have been reported presenting conclusive evidence that ABO incompatibility may be of pathologic importance during pregnancy.<sup>1</sup>

On the other hand, the antigenic properties of the A and B blood groups in pregnancies with ABO incompatibility have been demonstrated several times.

Jonsson<sup>2</sup> was the first to demonstrate the presence of potent anti A and anti B lysins in the mothers about three months after the delivery as a rather selective response to A or B antigen in the fetus.

Boorman and co workers<sup>3</sup> and Smith<sup>4</sup> have confirmed this by studying isoagglutinin titers. They found only a slight increase during pregnancy, but a rapid and marked increase was seen shortly after delivery. Smith found this increase of titer only in cases where the infants were secretors.

The discovery of incompatibility in the Rh system as the most common cause of hemolytic disease in the newborn infant gave rise to new problems such as: Why is Rh immunization during pregnancy not a more common occurrence in view of the fact that the possibility of Rh immunization so often exists? And another question: Is there any relation between Rh and A or B immunization?

With regard to the latter question Levine<sup>5</sup> has found a somewhat higher incidence of Rh immunization in ABO compatible pregnancies. Chown<sup>7</sup> and Davidsohn<sup>8</sup> found an increased AB incidence in Rh immunized women. On the other hand, Gurevitch and co workers<sup>9</sup> have published two cases where there apparently might have been a positive correlation between A and Rh immunization. In conformity with this Wiener<sup>10</sup> has found in Rh immunized women who have borne A or B children in ABO incompatible pregnancies a high anti A or anti B titer respectively.

This study, which is based upon blood samples from pregnant women, aims at an investigation of anti A and anti B isoagglutinin titers in Rh immunized women compared with adequate control material. The results thus obtained necessitated an investigation of the various anti Rh fractions. The work was carried out during the autumn of 1947.

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## MATERIAL AND METHODS

*Material*—The anti Rh containing sera which were examined were samples obtained from women in the last two months of pregnancy. Of 25,000 samples 87 contained Rh antibody.

*Methods for Routine Rh Determination*—The samples were tested for Rh in the usual manner. Cells from the coagula were suspended to 2 per cent in saline. One drop of the suspension was mixed in small test tubes with 1 drop of anti Rh serum. The tubes were incubated at 37° C for two hours, and the sediment then was examined for agglutination on slides under the microscope. Two different anti Rh testing sera were used parallelly, one containing anti D only and one containing anti D + C, both possessing good agglutinating power in saline media. Sera from all samples, including Rh positive ones, were tested by the same technique against a panel of Rh genotyped O cells. Three drops of serum plus 1 drop of a 2 per cent suspension of cells in saline were used. Sera giving dubious reactions and sera from Rh negative individuals to a great extent were examined also by Diamond's slide test or tested against cells suspended in serum and/or albumin and also partly tested by the Coombs' technique.

*Storage and Control*—The Rh containing sera were stored at -10° C, and groups of thirty sera then were tested. As a control for the isoagglutinins anti A and anti B, 100 sera from Rh negative pregnant women who were not immunized and 100 sera from Rh positive pregnant women were used. The control sera were picked out at random over a period of six months and were stored and handled in the same way as the sera containing Rh antibody.

*Determination of the Various Anti Rh Fractions Contained in Rh Sera*—The anti D fraction contained in the anti Rh sera was determined by titration both in serum and in saline media against O R<sub>1</sub>r cells suspended in serum and in saline respectively. The serum media used were mixtures of fresh O Rh+ sera from blood donors. The titration was performed in relatively large Rh test tubes with a relatively heavy calibrated capillary pipette graded to approximately 0.06 cubic centimeters. The same pipette was used for the distribution of dilution fluid and for the distribution of the suspension of cells. After incubation for two hours at 37° C the test was read microscopically.

The sera were also tested against O R'r and O R''r cells to ascertain the content of anti C and anti E agglutinins. Two drops of the serum were mixed with 1 drop of the cells suspended in saline. All of the eighty seven anti Rh sera also were tested to ascertain the content of anti C\* agglutinin, all sera being parallelly tested against O R<sub>1</sub>\*r and O R<sub>1</sub>r cells in which D antigen was blocked. Blocking was performed with a serum containing anti D + E exclusively in incomplete form (blocking serum taken post partum after the third stillbirth in an Rh negative woman who was married to an R<sub>2</sub>r or R<sub>2</sub>R man). One volume of washed, packed O R<sub>1</sub>\*r and O R<sub>1</sub>r cells was mixed with 50 volumes of a 20 per cent blocking serum in saline, incubated for fifteen minutes at 37° C, centrifugated and washed with saline once, and then suspended to 2 per cent in saline. One drop of this suspension was added to 2 drops of anti Rh serum. Otherwise the usual technique was used.

*Determination of Anti A and Anti B Isoagglutinins*—The anti A and anti B isoagglutinins in all three groups were then determined in parallel series against the same Rh positive A<sub>1</sub> and B cells from fresh coagula. The cells were suspended to an approximately 1 per cent suspension in isotonic saline. The titrations were performed in successively doubled dilutions in test tubes. The volumes of each serum dilution and cell suspension were 0.1 cubic centimeter. The test tube racks were then placed overnight at +5° C and afterwards for one hour at room temperature. The reading of end point titers was done microscopically against a strongly illuminated white background, direct illumination on the tube was avoided.

## RESULTS

For calculating the titer results the log<sub>2</sub> of the conventional titer values was used (titer step values). The sera agglutinating cells in the first tube only

then have the titer value 1. In Tables I and II the arithmetic averages for the titer values in the various groups are recorded with the deviation of the means

$$\mu = \sqrt{\frac{\sum (X - \bar{X})^2}{(n-1)n}}$$

Where  $X$  is the single observation in the group

Where  $\bar{X}$  is the arithmetic average in the group

Where  $n$  is the number of observations in the group

Whether differences between the average values had any real statistical importance was tested with the formula  $t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\mu_1 + \mu_2^2}}$

which is the difference divided by the deviation of the difference. That  $t$  is greater than 3 was taken as a sign that the difference is real (not due to chance).

Furthermore, some correlation coefficients ( $r$ ) were calculated even though the material really was too small for such an analysis

$$r = \frac{\frac{1}{n} \sum X U - \bar{X} \bar{U}}{\sigma_X \sigma_U} \quad \text{where } \sigma_X = \sqrt{\frac{\sum (X - \bar{X})^2}{n-1}}$$

is the deviation of  $X$  in the group

*The Antibody Content in the Sera*—Prozone phenomenon against O R<sub>0</sub> 1 cells in saline medium was seen in five sera, that is no agglutination in the first or in the second and third tube no prozone in serum medium. A relatively large number (four) of the sera contained anti D+C+E. For three of these sera some kind of family control was obtained. Rh negative multiparous married to men of the genotype C+, D+, E+, e+

TABLE I. ANTI D TITERS AND THE CONTENT OF ANTIC C E IN ANTI RH SERA AND THE DISTRIBUTION TO THE ABO SYSTEM

ABO TYPE	TYPE OF Rh ANTIBODY	NUMBER	AVERAGE OF ANTI D TITERS			
			NaCl MEDIUM		SERUM MEDIUM	
A	Anti D only	23	239±	0.50	$t = 2.80$	491± 0.74
	Anti D+C	9	$t = 1.68$	$t = 3.85$	$t = 1.74$	664± 0.65
	Anti D+C+E	6				
	Anti D	4				
	Anti D+C	3	355±	0.47		
O	Anti D only	15	253±	0.62	$t = 1.66$	413± 0.73
	Anti D+C	11	$t = 1.64$	$t = 3.19$	$t = 2.60$	689± 0.76
	Anti D+C+E	6				
	Anti D	1				
	Anti D+C	1	389±	0.54		
B	Anti D only	5	340±	1.25		360± 1.16
AB	Anti D only	2	10±	0.0		266± 1.66
	Anti D+C	1				

The distribution of the anti-Rh-containing sera to the ABO and MN systems was within normal limits

The effect of the serum medium on the anti-D titer values is so marked in the complex anti-Rh sera (those containing anti-D+ C, C<sup>w</sup>, E) that the difference between the anti-D titer values in serum medium and saline medium is of statistical importance. The correlation between titer values for anti-D in serum medium and in saline medium is determined by the following correlation coefficients

$$\begin{aligned} \text{In A blood} \quad r_{\text{anti-D NaCl, anti-D serum}} &= +0.34 \\ \text{In O blood} \quad r_{\text{anti-D NaCl, anti-D serum}} &= +0.60 \end{aligned}$$

TABLE II AVERAGE TITERS FOR ISOAGGLUTININS ANTI B AND ANTI A

ABO TYPE	TITER	GROUP I Rh NEGATIVE WITH Rh ANTIBODIES	GROUP II Rh POSITIVE WITHOUT Rh ANTIBODIES	GROUP III Rh NEGATIVE WITHOUT Rh ANTIBODIES
A	Anti B	6.06	7.12	7.51
	$\mu$ Number	0.25 45	0.15 51	0.18 49
O	Anti B	6.41	7.40	7.73
	$\mu$ Number	0.25 34	0.15 40	0.19 41
	Anti A	7.79	7.60	7.81
	$\mu$ Number	0.30 34	0.16 40	0.16 41
B	Anti A	7.20	7.89	7.70
	$\mu$ Number	0.37 5	0.24 9	0.25 10

Table II shows that the anti-B titers in A and O blood in Rh negative women with Rh antibody are considerably lower than in the control group, while the anti-A titers in O and B blood show no noticeable difference between the groups. The difference in anti-B titers was tested by the method previously outlined.

#### ANTI B TITERS IN A BLOOD

Group III	against I	t 4.67
Group II	against I	t 3.63
Group III	against II	t 1.63

#### ANTI B TITERS IN O BLOOD

Group III	against I	t 4.55
Group II	against I	t 3.72
Group III	against II	t 1.38

Thus it appears that the low anti-B titers in Rh-negative women with Rh antibody compared with the control groups give differences which are too large to be caused by chance. A similar testing of the anti-A titers in O blood gave values less than 1. The low anti-B titers in Rh-negative, Rh-immunized women suggests that there may be an antagonism between anti-B and anti-Rh, and furthermore, a possible negative correlation between the anti-B titers determined against B Rh<sup>+</sup> cells and the anti-D titers determined against O R<sub>0</sub> r cells. The correlation coefficient for the connection between anti-B titers and anti-D titers in saline medium, and between anti-B titers and anti-D titers in serum medium was calculated therefore, even though the material was too small for such an analysis.

#### CORRELATION COEFFICIENTS (r) IN

	A BLOOD	O BLOOD
$r_{\text{anti-B anti-D in NaCl medium}}$	+ 0.43	+ 0.02
$r_{\text{anti-B anti-D in serum medium}}$	- 0.05	+ 0.11



That means that a simple negative correlation between anti B and anti D titers cannot be demonstrated

#### COMMENT

It is possible that the average age of Rh negative, Rh immunized women is somewhat higher than that of the control group. It is a fact that the isoagglutinin titers decrease with increasing age after adult life. Titrating the anti B in about 1,200 women of various ages. Hutmayer<sup>11</sup> found a decrease of the average titer of approximately one third titer step among women between 18 and 50 years of age. The anti A titers were determined in the same way in about 700 women, and the decrease with age in titer values paralleled the decrease in anti B titer. The anti B titer in Rh negative Rh immunized women is a whole titer step lower than the anti B titers in the control group while the corresponding anti A titers show neither systematic nor definite statistical difference. A possible difference in age in the three groups does not therefore, explain why the anti B titer is so low in Rh negative Rh immunized women.

#### SUMMARY

Eighty seven antenatal anti Rh sera were tested to ascertain the ABO MN distribution and the content of various fractions of Rh antibody and the anti B and anti A isoagglutinin titers.

The ABO and MN distribution was within normal limits. Lower anti B titers are found in Rh negative women with Rh antibodies compared with Rh negative women without Rh antibodies and also compared with Rh positive women. The difference (one titer step) is too large to be caused by chance. A possible negative correlation between anti D and anti B titers cannot be demonstrated.

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# FACTORS IN THE USE OF MERCURIC BICHLORIDE FOR BIOLOGIC STUDIES

WITH ESPECIAL REFERENCE TO BLOOD COUNTS

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HAYEM<sup>1</sup> first used the solution which has come to be designated by his name in order to meet his need of a diluent for experimental studies with blood. He simply adapted to his needs a fixative which he was accustomed to use for tissues in general. Its widespread adoption came about during the early phases of quantitative blood studies before demands for stringent accuracy had been formulated. Various workers have found inadequacies of distribution in its use and have offered formulas for other diluents as well as suggestions to lessen this difficulty with Hayem's solution. The errors in distribution in the use of the solution usually have been attributed to clumping or balling of cells in the diluting pipettes though Joergensen<sup>2</sup> also found unequal distribution on the counting chamber filled in the manner then in vogue. The clumping usually was ascribed to the age of the diluent and to a precipitate that may occur with age. Upon development of their mechanical rotor for mixing blood, Bryan and Garrey<sup>3</sup> found that while avoidance of these factors represents important precautions, it is safer to use a diluent for red blood counts that does not contain bichloride. They suggest the use of Toison's solution. They found the distribution of cells to be very exact when the rotor was used for white counts. Clumping does not occur in white counts. Ch'u and Forkner<sup>4</sup> also found that the presence of bichloride in the diluent causes clumping in certain pathologic blood, even when the pipettes are shaken by hand. They suggest that the clumping is due to the effects of the bichloride on the plasma proteins, but they did not investigate the problem from that angle and confined their studies to substitution of a different diluent, Gower's solution. These as well as other suggestions for substitution of different diluents never have received general adoption, and Hayem's solution has continued to be the diluent in common use without regard for the dangers of distribution inherent in its use. The causes underlying the inequalities of distribution in the use of bichloride for diluents were not investigated. Examination of the causes was, therefore, undertaken in an effort to so modify the formula of Hayem's solution as to avoid the dangers inherent in it, rather than to attempt further substitution of a different diluent.

It was found that satisfactory modification of Hayem's formula is possible from either of two approaches. (1) the content of bichloride can be reduced to low as that in Joergensen's solution, together with careful regulation of the pH

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within a narrow zone or (2) a protective substance, such as gelatin or lecithin can be added to the formula for Hayem's solution without consideration of pH up to 7.0. Use of gelatin was found to be the more practical method. The advantages of its use: preparation of the solution and comparison between counts made with it and with Hayem's solution without gelatin or with Jorgensen's solution have been published briefly. The studies that follow are those that were carried out to determine the causes that underlie the clumping of blood with diluents that contain bichloride and the factors that operate in the prevention afforded by the methods found. They are presented not only because of their pertinence to the use of diluents which contain mercuric bichloride for blood counts, but also because they indicate the significance of the pH and ionic content of diagnostic and therapeutic agents in the precipitative, agglutinative and colloidal phenomena of the blood.

### METHODS

The studies required approach from several aspects and it seems preferable therefore on the whole, to describe the methods employed as each group of findings is presented. Consequently only those procedures will be described at this point which were used routinely to determine the data for each set of experiments.

All of the studies were made upon the blood of a single individual. Except where the studies were made on fractions of whole blood all studies were made by drawing blood into automatic pipettes, both Trenner and Hual from a freely flowing finger stick and immediately diluting with the desired solution. The pipettes were shaken a few times by hand and then rotated for thirty minutes or longer on a commercial model of the Bryan Garvey rotor. Control studies made with one of the original rotors were entirely similar. The gentleness and smoothness of the movements of the rotor together with the fact that it mixes in fixed planes, permit detection of very slight degrees of precipitation or aggregation and therefore facilitated in the elucidation of the underlying factors that feature in the occurrence and prevention of clumping in blood counts.

The occurrence of precipitation, aggregation, clumping and so on in the pipettes was determined by microscopic inspection of counting chambers filled as for counts. Levy Hauser chambers with improved Neubauer ruling were used.

The determinations of the hydrogen ion concentrations of the solutions were made by means of a Hellige pH meter to which were attached the glass electrode and calomel tube of a Beckmann meter. Solutions of HCl and NaOH were used for adjustments of pH. Glass weighing bottles were used as containers for the solutions and the pipettes were filled directly from them immediately after adjustment. The fluid from the pipettes was in turn expressed directly into cups when the pH of the contents was to be determined.

### PRESENTATION AND DISCUSSION OF EXPERIMENTAL DATA

The experimental data are presented in Tables I to IV and in Fig. 1. The values for pH stated in the following studies and recorded in the tables represent the pH of the diluents before they were mixed in the pipettes with the blood or fractions thereof. The pH readings of the resultant mixtures were modified according to the buffering action of the different biologic fluids and curves are given in Fig. 1 to show the readings of the mixtures under various experimental conditions. The pH readings of the original diluents before experimental adjustments were as follows: Hayem's solution, 5.3; Hayem's solution plus gelatin, 4.9; Jorgensen's solution, 5.3; saline base, 6.0. All pH values

are recorded, approximately, to the nearest 0.5 reading. Blank recordings indicate lack of data at those values. Absence of reaction (that is precipitation, aggregation, or clumping) at any particular value is tabulated as zero. The presence of reaction at any pH reading is indicated by a plus sign. It does not seem important to indicate the degree or qualitative characteristics of the reaction since these are regulated, in large part, by combinations of factors such as the physical characteristics of any particular precipitation and its capacity to be rolled into balls and to catch cells, by the presence or absence of plasma or serum in any particular test of the series, by surface forces of the cells,

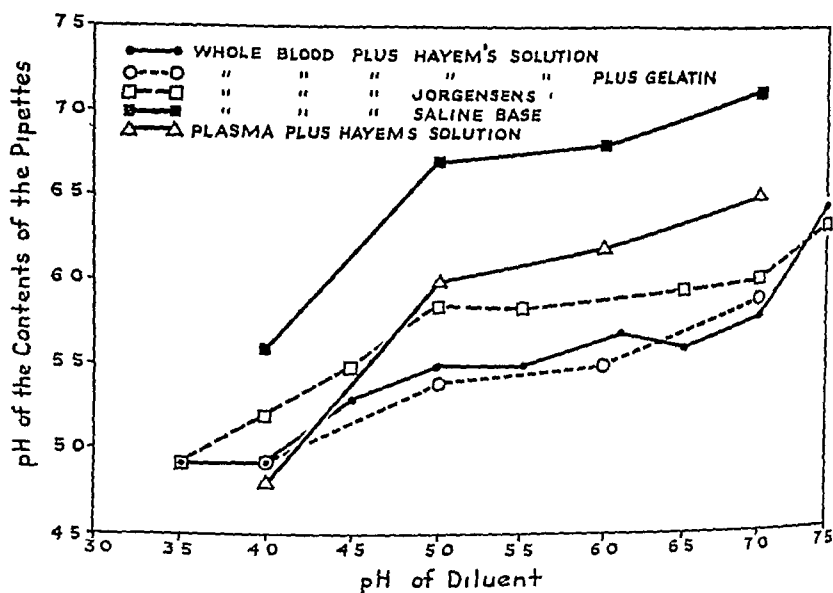


Fig 1—Change in pH of diluents by admixture with whole blood or plasma

irrespective of precipitation within the plasma, and so forth. It is these factors which are often counteracted by vigorous shaking with the result that the underlying precipitation or aggregation is obscured. Therefore only the presence or absence of reaction in the pipettes at any given pH with any given diluent is tabulated, without record of the physical state of the reaction. The latter will be described and discussed where it seems significant to the understanding of the factors involved in its formation. Differentiation between certain terms used in these discussions may be aided by definition at this point. The term precipitation is employed to indicate the occurrence of diffuse, fine, crystalline particles throughout the menstruum. The terms aggregation and agglutination are employed synonymously to indicate close adherence of erythrocytes to each other without adhesive mechanism that is microscopically visible. The two states differ, apparently, only in the number of cells adherent to each other. Agglutination apparently represents aggregation of sufficient cells to cause settling of the masses. The terms clumping and balling are used synonymously to indicate the conditions usually met in whole blood when cells and precipitate unite in fibrous masses.

*Action of Bichloride as a Fixative*—Studies made by the addition of other unrelated tissue fixatives to the saline base of Hayem's or Jørgensen's solution indicate that the clumping effect on blood of diluents that contain bichloride (Fig 2, 3 to 6) is related to its action as a biologic fixative. The solutions to be tested were mixed with whole blood in pipettes and rotated for a short period in the manner used in making blood counts with Hayem's or Jørgensen's solution. The saline base alone caused no clumping and only questionable lysis at the hydrogen ion concentrations under study (Table I). Addition of absolute alcohol to the saline base in dilutions up to 90 per cent by volume caused lysis, above that it caused clumping similar to the clumping observed with Hayem's solution. The same statements hold for solutions of saline base and acetone. They caused lysis in dilutions up to 55 volumes per cent and clumping above that proportion. Formalin caused lysis when the pH was below 5.0. Above that, formalin caused neither lysis nor clumping in concentrations between 10 and 60 volumes per cent. Concentrations above that caused clumping. In similar manner a balance was found to exist between hemolysis

TABLE I. EFFECT OF pH ON CLUMPING OF WHOLE BLOOD WITH DIFFERENT DILUENTS

DILUENT	pH											
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	
Jensen's with 25% HgCl <sub>2</sub> *					L	L	L	L	L			
					0	0	0	0	+	+	+	
1h 50% HgCl <sub>2</sub>					L	L	L	L				
					+	+	+	+	+	+	+	
1h 10% HgCl <sub>2</sub>			L	L	L							
			+	+	+	0	0	0	+	+		
1h 100% HgCl <sub>2</sub>			L	L								
			+	+	0	0	0	0	+			
1h 1% HgCl <sub>2</sub>		L	L									
		+	+	0	0	0	0	+	+			
1h 150% HgCl <sub>2</sub>			+	+	0	0	0	0	+			
1h 100% HgCl <sub>2</sub> plus gelatin		0	0	0	0	0		0	0	+	+	
Hayem's		+		+	+	+			+			
Hayem's gelatin	0	0	0	0	0	0	0	+	+			
Hayem's base		L†										
		0	0	0		0	0		0		0	
Hayem's gelatin		0	0						0		0	
Hayem's base plus 2 drops Na <sub>2</sub> WO <sub>4</sub> †	+	+		0	0				0		0	
Hayem's 2 drops Na <sub>2</sub> WO <sub>4</sub>	+			+						+		
Hayem's 10 drops Na <sub>2</sub> WO <sub>4</sub>	+	+		+		+	+	+	+	+	+	
Hayem's 10 drops Na <sub>2</sub> WO <sub>4</sub> plus gelatin		+		+	+	+	0	0	0			

+, Positive reaction in the form of precipitation aggregation agglutination or clumping  
 0, opalescence but no frank precipitate L, some degree of lysis 0 no visible reaction  
 blank, no studies made

The percentages of HgCl<sub>2</sub> recorded with the dilutions of Jørgensen's solution refer to the amount of HgCl<sub>2</sub> used compared with the normal amount for Jørgensen's solution

† All solutions of Na<sub>2</sub>WO<sub>4</sub> were made by use of the same dropper and a 10 per cent stock solution

and clumping, dependent upon the proportions of bichloride added to the saline base (Table I). When added in the proportion of 25 per cent of the amount in Jorgensen's solution (0.012 Gm. to 100 cc.) lysis alone occurred below pH 7.0, and clumping above. In a proportion of 50 per cent of that in Jorgensen's solution both lysis and clumping occurred when the pH was below 7.0, and clumping only at higher values. When the content was 75 per cent of that in Jorgensen's solution lysis accompanied by clumping occurred only below pH 6.0, while clumping alone occurred above pH 7.0. The narrow zone between these values was neutral as far as these effects were concerned—neither lysis nor clumping occurred. With full strength Jorgensen's solution, that is 0.05 Gm. per 100 cc., the neutral zone was extended somewhat on the acid side but otherwise the events were similar to those with 50 per cent concentration. When the concentration was 125 per cent of that in Jorgensen's solution the neutral zone was extended still more to the acid side, while the events in the basic direction remained unaltered. At a concentration of 150 per cent of that in Jorgensen's solution, lysis ceased and clumping alone occurred on both sides of a neutral zone, and the reactions became more like those obtained with Hayem's solution. With the latter, lysis did not occur at the pH values tested, but clumping occurred at all pH levels, with a tendency, however, to a neutral zone in the same region as the neutral zones with the weaker solutions.

It seems obvious therefore that many, if not all, fixatives function in two ways. If sufficiently dilute they cause more or less lysis, which may or may not be accompanied by clumping of cells that have not been destroyed, the liability to lysis is influenced not only by dilution but also by the pH of the diluent. If sufficiently concentrated the fixatives no longer cause lysis but exert only a clumping action. Experimental explanation of these actions, as far as bichloride is concerned, is presented in the groups of studies that follow.

The danger of lysis as well as of clumping in the use of Jorgensen's solution as a diluent for red counts, unless the pH is carefully controlled, is obvious. The clinical significance of this is discussed elsewhere.\*

*The Relationship of the pH of the Diluent to Clumping in Whole Blood (Table I)*—As stated previously, addition of lecithin or gelatin was found to prevent the clumping inherent in the use of Hayem's solution (Fig. 2, compare 2 and 7 with 1, 3, 4, 5, and 6). The factors involved in this protective action

Fig. 2—The low-power magnifications were obtained with a 16 mm objective and a  $\times 15$  ocular. The high-power magnifications with a 4 mm objective and  $\times 10$  ocular. The diluting pipettes were rotated for thirty minutes beginning immediately after filling. 1. Illustration about the best distribution obtainable with standard unadjusted Hayem's solution. Even in this case the tendency toward aggregation of cells is demonstrable in the form of thin groups scattered here and there. The pH of the Hayem's solution was 4.8. 2. Distribution with Hayem's solution containing gelatin. The mixture had been left in a Pyrex flask for many months. Aggregation of cells did not occur. The pH of the mixture was 4.0. 3. Detail of the aggregates in preparation made with Hayem's solution adjusted to pH 3.1. Small masses of cells aggregate rather tightly. Little precipitate occurs in the plasma and if present, it rarely becomes intermingled with the aggregates of cells. 4. Same solution as 1, but from a different pipette. The tendency toward aggregation of cells is more pronounced in this preparation. 5. Same as 4. Detail of the aggregates in preparations made with standard unadjusted Hayem's solution. Only a few cells occur in each aggregate and they are loosely balled with a soft amorphous precipitate. 6. Detail of the aggregates in preparations made with Hayem's solution adjusted to pH 7.2. Cells agglutinate into large tight masses and these become enmeshed with large amounts of refractive fibrinous precipitate. 7. Same as 2. Detail of preparations made with Hayem's solution containing gelatin. There is no tendency toward aggregation of cells and any precipitate remains scattered.

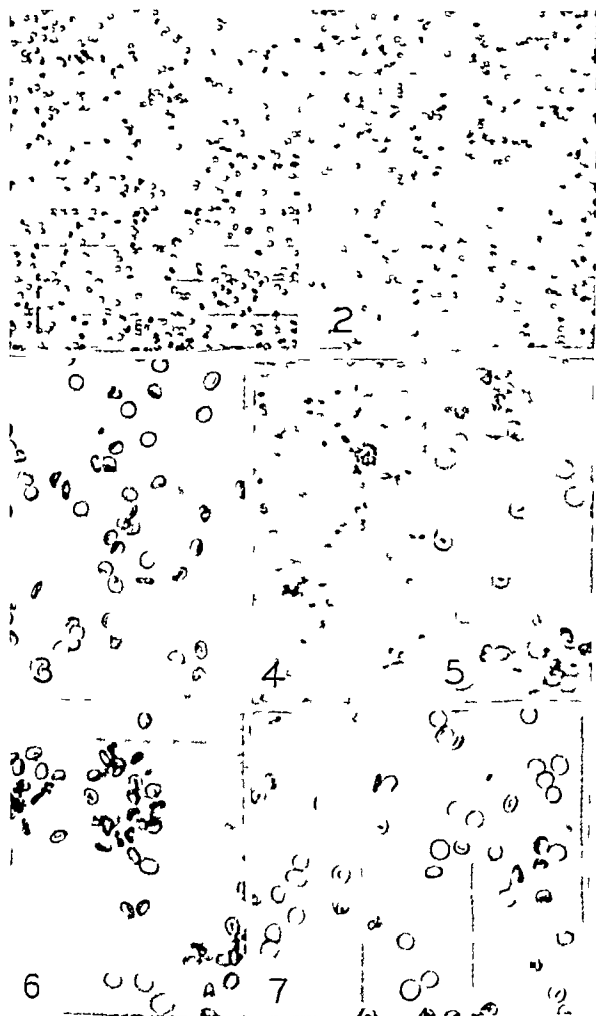


Fig. 9

(See opposite page for legend)

have been studied in detail for solutions containing gelatin. The gelatin was added in the proportion of 0.01 Gm. to 100 cc. of Hayem's solution. The method of preparation and the precautions have been published.<sup>5</sup>

It already has been shown that Hayem's solution caused clumping of the blood at all pH levels that were studied. It may be seen from Table I that when the solution contained gelatin, however, clumping was inhibited below pH 7.0. If the proportion of bichloride in the diluent was reduced to that in Jorgensen's solution (as was also discussed in the preceding section) clumping occurred on both sides of a neutral zone, between pH 5.0 and 7.0, in which neither clumping nor lysis occurred. In addition to clumping, lysis of some of the cells occurred on the acid side of that zone. As the concentrations of bichloride were reduced, lysis and clumping on the acid side of the neutral zone took place at increasingly greater pH, with consequent narrowing of the zone. As the concentrations of bichloride were increased, lysis and clumping on the acid side of the neutral zone occurred only at increasingly lower pH, with consequent widening of the zone. When the concentration was sufficient, lysis did not occur at all and the reaction became similar to that with Hayem's solution. It may be seen from Table I that gelatin afforded the same protective action against clumping with the weaker solutions of bichloride that it did with Hayem's solution. In addition it also protected against the lysis caused by weak acid solutions of bichloride. The studies with these weaker solutions reveal, therefore, something of the character of the clumping effect of bichloride better than those made with a concentration as great as that in Hayem's solution. They bring out the fact that clumping with bichloride is more intense on both sides of a middle zone of pH and that when the solution is weak enough a neutral zone at which clumping does not occur becomes definite. Addition of gelatin is effective only on the acid side of that zone.

In addition to the preceding findings with the solutions of bichloride, there also were observed qualitative, though not necessarily quantitative, differences in the character of the clumping at different levels of pH. At very low values the erythrocytes aggregated into groups that seemed free of any surrounding material (Fig. 2, 3). At higher values they also often were aggregated, but in addition they were always enmeshed or embedded in an amorphous or fibrous background (Fig. 2, 5 and 6). This background varied in appearance from a faintly amorphous character at the lower pH readings to a coarse, reactive, fibrous nature at the higher readings. The more fibrous the appearance, the more scattered were the cells in and about the background so that the clumps took on the appearance which is commonly observed when clotting has been allowed to occur before addition of the diluent.

When sodium tungstate was substituted for bichloride in the saline base and this diluent was used with whole blood in the same manner as the solutions containing bichloride, clumping occurred at all pH values when the metallic salt was concentrated, but only at pH 4.0 or below when it was weak (Table I). Similar to the reaction in solutions containing bichloride, addition of gelatin to the diluent prevented clumping in one direction of pH, but in contrast to its



effect in solutions containing bichloride it prevented clumping in the basic instead of the acid direction. In other words, strong and weak concentrations of sodium tungstate exerted an effect on whole blood analogous to that of bichloride, except that the pH level at which clumping occurred irrespective of the presence of gelatin, was reversed to the acid side. In addition, lysis was not detected under any of the experimental circumstances. These reactions between whole blood and sodium tungstate will be discussed further under Correlation and Discussion of Experimental Findings.

In summary, these studies reveal the following facts: first with concentrated solutions containing bichloride clumping of cells occurred at all pH levels but the character of the clumping varied; second with weaker solutions of bichloride a neutral zone was present at which clumping did not occur, but variable degrees of lysis, as well as clumping occurred on the acid side of that zone and clumping only on the basic side; third solutions of sodium tungstate, instead of bichloride, acted in a manner similar to solutions of bichloride except for reversal of the critical level of pH; clumping occurred at all levels with concentrated solutions, but on the acid side only with weak solutions; fourth, gelatin gave protection from the clumping of blood with solutions of mercuric bichloride below pH 7.0 or sodium tungstate above pH 6.0. It also protected the cells from lysis with weak acid solutions of bichloride.

It is obvious that clumping of the blood is associated with the presence of metallic salts and is prevented by the addition of gelatin at certain ranges of pH, the range varying with the salt employed. The following studies were carried out to determine the part played by the different fractions of the blood in the clumping and the nature of the forces that respond to pH and of those that are inhibited by gelatin.

*Studies With Whole Washed Erythrocytes (Table II)*—Blood for these studies was collected by venous puncture in tubes containing either dried sodium citrate or 1 drop of a concentrated solution of sodium oxalate. The tubes were centrifuged and the plasma was collected separately for studies to be described later. The cells were washed with about 10 volumes of physiologic saline centrifuged, separated, and rewashed until the process had been repeated six or more times. The erythrocytes were then suspended in equal volumes of saline and the suspension was used with the pipettes and various diluents in the manner and under the conditions described in the studies with whole blood. These plasma free cells were found to aggregate, or agglutinate not only with Hayem's solution but also with all dilutions of Jorgensen's solution employed almost irrespective of the pH. The aggregation was similar to that observed with whole blood in Hayem's solution at very low pH values (Fig 2, 3). That is the aggregates appeared free of any amorphous or fibrinous background. The aggregation was inhibited by gelatin below pH 6.5. With sodium tungstate, on the other hand aggregation of erythrocytes occurred only on the acid side and was unaffected by gelatin.

It is obvious from these findings that while the fibrinous qualities in the clumping of whole blood with bichloride are related to the presence of plasma, aggregation of the erythrocytes is independent of the plasma. Furthermore,

it is obvious that plasma actually gives protection from aggregation in the neutral zone when the metallic salt is weak enough. Gelatin acts as a more potent protective agent and protects even with strong solutions of the metallic salt.

Lysis did not occur with the weak solutions of bichloride as it did with whole blood. It seems probable that the more fragile cells had been lysed automatically in the process of washing and that the remaining cells were sufficiently resistant for the conditions of the experiment.

*Studies With Lysed, Washed, Erythrocytes (Table II)*—The saline suspensions of erythrocytes that were used for the preceding studies with whole washed erythrocytes were centrifuged and freed of as much saline as possible. They were lysed by the addition of about twice their volume of distilled water. The solutions were centrifuged and the supernatant fluid was filtered through

TABLE II EFFECT OF PH ON CLUMPING OF WASHED RED BLOOD CELLS AND FILTERED LASED RED BLOOD CELLS WITH DIFFERENT DILUENTS

DILUENT	pH									
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0
<i>Washed Red Blood Cells</i>										
Jorgensen's with 25% HgCl *		0				+				+
With 100% HgCl gelatin		0				0				0
With 100% HgCl		+		+		+		+		+
With 100% HgCl plus gelatin		0		0		0		0	+	
Hayem's	+	+			+	+				+
Plus gelatin	0	0			0	0	+	+	+	
Saline base plus 5 drops Na2W04†		+						0	0	
Plus 5 drops Na2W04 plus gelatin		+						0		
Saline base										0
<i>Lysed Red Blood Cells†</i>										
Jorgensen's with 25% HgCl	+					+				+
With 100% HgCl	+		+	+		+	+	+	+	+
Drawn to automatic stop only	?					+				
With 100% HgCl plus gelatin	?			?		?		+	+	
Hayem's	+	+	+			+		+		
Plus gelatin	+			+				+		
Saline base plus 5 drops Na2W04		+	+	0		0				
Saline base		0				0				

+ Positive reaction in the form of precipitation aggregation agglutination or clumping  
 ?, opalescence but no frank precipitate 0 no visible reaction blank no studies made  
 \*See footnote \* Table I  
 †See footnote † Table I  
 ‡The fluid for these tests was drawn to about one tenth the volume of the pipette instead of to the automatic stop

No 5 Whatman paper No membranes could be found by microscopic examination after this treatment The filtrate was subjected to tests similar to those employed with whole erythrocytes If sufficient of the filtrate was used, it was precipitated by either Hayem's or Jorgensen's solution at all hydrogen ion concentrations studied, and the precipitation was not prevented by the presence of gelatin in the diluent in the usual proportions With sodium tungstate, precipitation occurred up to but not beyond pH 4.5 There was no difference between the reactions of the erythrocytes that had been procured by the use of oxalate and those procured by the use of citrate

The unprotected, washed erythrocytes obviously had reacted to the diluents in accord with the reactions of their contents to the same diluents but they could be prevented from certain of the reactions by the presence of gelatin while the contents were not thus protected from reaction It would seem therefore, that gelatin must function by protecting the surface of the erythrocytes from contact with the metallic salt but not beyond certain critical pH values These values vary according to the nature of the metallic salt

*Studies With Plasma and Serum*—The plasma and serum for these studies were obtained at the same time as the cells used in the preceding studies The plasma used was from the oxalated specimen only These fluids were subjected to tests similar to those made on blood and cells As in the case of the contents of lysed erythrocytes if sufficient plasma was used it was precipitated at all hydrogen ion concentrations tested by either Hayem's or Jorgensen's solution and this action was not prevented by the presence of gelatin in the usual proportion In order to simulate the conditions that exist in making blood

TABLE III EFFECT OF pH ON PRECIPITATION OF PLASMA OR SERUM WITH DIFFERENT DILUENTS

DILUENT	pH										
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5
<i>Plasma</i>											
open s with 0.5% HgCl <sub>2</sub> *		0				0			0		
th 100% HgCl <sub>2</sub>	0	0	0	+	+	+			+		
m s		0				+			+		
is gelatin		0				+			+		
se base plus 5 drops		+				+				0	
W04†											
as 5 drops N12W04 plus		+				0			0		
gelatin											
se base	0					0				0	
<i>Serum</i>											
ensen s with 0.5% HgCl <sub>2</sub>		0				0			0		
th 100% HgCl <sub>2</sub>	0	0	0	0	0	0	+	+	+		
m s		0				+			+		
is gelatin		0				+				+	
se base plus 5 drops		0				0				0	
W04											

+ Positive reaction in the form of precipitation aggregation agglutination or clumping  
 0 no visible reaction blank, no studies made.

See footnote Table I

† See footnote † Table I

counts, therefore, studies were also carried out on plasma drawn only to the automatic stop of the pipette (Table III). Under those circumstances it was not precipitated below pH 5.0 by the solutions of bichloride or above pH 6.0 by those of sodium tungstate. As was the case when larger amounts of plasma were employed, these precipitations with lesser amounts also were not prevented by the presence of gelatin in the usual proportions.

The findings with serum varied from those with plasma in two respects: the serum was not precipitated as easily nor at as low a pH with the weak solutions of bichloride as was the plasma, and it was not precipitated at all by the amounts of sodium tungstate that caused precipitation of the plasma.

#### CORRELATION AND DISCUSSION OF EXPERIMENTAL FINDINGS

With amounts equivalent to those present in blood counts, plasma was not precipitated by Hayem's solution below the isoelectric point of any of the usual plasma proteins. Precipitation did occur above that point, but the precipitation was at first soft and slight and fluffy, and it was only as the pH rose to levels above the isoelectric point of most of the common plasma proteins that precipitation became sturdy and abundant enough to ball with rotation. The same statements may be made of the reactions to Jørgensen's solution. Gelatin did not affect these precipitations. Yet gelatin prevented clumping in whole blood at hydrogen-ion concentrations equal to those at which precipitation of plasma proteins occurred. Obviously the clumping in whole blood is due to factors other than just the action of the metallic salts on the plasma proteins alone.

The washed erythrocytes aggregated, or agglutinated, at all pH levels with any dilution of the mercurial, and this could be prevented with gelatin up to pH 6.5. Yet if weak solutions of the mercurial were used with whole blood, clumping did not occur in a neutral zone. Therefore the plasma proteins gave protection from aggregation despite the fact that they themselves were not prevented from precipitation at this hydrogen-ion concentration. Again, obviously, the plasma proteins are not the entire cause of clumping in whole blood, and they are even somewhat protective, though less so than gelatin.

In spite of the presence of the plasma proteins then, clumping occurs in whole blood at all pH levels with concentrated bichloride solutions, and on either side of a neutral zone with dilute solutions. The clumping with both the weak and strong solutions of bichloride is prevented up to a certain critical pH of 7.0 by the addition of gelatin. Since the washed erythrocytes aggregated, or agglutinated, at all hydrogen-ion concentrations, even with the dilute solutions of bichloride, and the aggregations could be prevented up to pH 6.5 by the addition of gelatin, it seems obvious that the factors which cause aggregation of erythrocytes in the presence of the metallic salt also regulate the clumping in whole blood with this salt.

The contents of the lysed erythrocytes precipitated at all pH levels and this was not prevented by gelatin. The contents were obviously subject to reactions similar to those which characterize plasma proteins. Without gelatin the washed erythrocytes responded much as their contents. Yet gelatin protected them from aggregation up to pH 6.5. It is obvious that intact erythrocytes

exert a somewhat different or more concentrated force than that of free proteins, and it is this which can be prevented up to a certain point by the protective agent, gelatin. They apparently function, when whole as large electro-negative colloids which aggregate in the presence of positive ions.

Granted that they do function as electronegative colloids the aggregation of erythrocytes in whole blood with weak solutions of bichloride on the far acid side of a neutral zone in which aggregation did not occur can be explained on the basis of colloidal aggregation due to the presence of sufficient positive electrolytes.

The occurrence of agglutination on the basic side of the neutral zone in contrast to that on the acid side, may be due to the fact that the gelatin itself is precipitated at this pH (Table IV). On the other hand it may be due to direct reaction between the bichloride and the contents of the erythrocytes at a pH concentration above the isoelectric point of hemoglobin. Gelatin did not prevent precipitation of plasma proteins at pH concentrations above their isoelectric points, even when those concentrations were less than the pH at which gelatin could be inactivated by precipitation. It seems probable therefore, that the aggregation of erythrocytes at pH 7.0 in whole blood or 6.5 with washed cells, was due to the inherent character of the cellular contents rather than to the precipitation of the gelatin. The studies with sodium tungstate and washed erythrocytes seem to support these conclusions. Colloidal aggregation above a pH at which direct union could occur between the metallic radical and the proteins within the cells was apparently suppressed by the presence of negative electrolytes. The explanation of the capacity of concentrated solutions of sodium tungstate to cause clumping in whole blood at high pH concentrations and of the protection afforded by gelatin, in contrast to this reaction with washed erythrocytes is not clear.

Whenever aggregation of erythrocytes occurs at pH levels at which precipitation of the plasma proteins may also take place the aggregation is accompanied by a background of more or less amorphous or fibrous material. It would seem that while the precipitated plasma proteins are not in themselves sufficient to initiate clumping they do qualify and intensify it if aggregation

TABLE IV EFFECT OF pH ON THE DILUENTS ALONE

TABLE IV EFFECT OF pH ON THE DILUENT												
DILUENT	pH											
	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	
ne base plus gelatin	0	0		0		0	0		0			
ne base plus gelatin plus Na <sub>2</sub> WO <sub>4</sub> *			+	+	0							
ne base plus gelatin plus Jørgensen's solution	0		0			0		0	0			
ne base plus gelatin plus Hayem's solution							0	+	+			
ensen	0	0		0		0		0	0		+	
em							0	+	+	+	+	

+ Positive reaction in the form of precipitation aggregation agglutination or clumping  
0 no visible reaction blank no studies made.

\*See footnote † Table I

or agglutination of the erythrocytes occurs. It is by this inhibition from aggregation that more vigorous shaking or the presence of gelatin acts to prevent clumping until critical pH levels occur.

That the clumping with weak, dilute solutions of bichloride at pH levels below those at which the plasma proteins precipitate was, nonetheless, accompanied by a fibrinous background is probably due to the lysis that occurred at those levels with liberation of cellular contents which could precipitate at such low levels. The precipitate then would have balled with the aggregated erythrocytes in the same manner as do precipitated plasma proteins when they occur in the presence of agglutination.

In summary then, these studies seem to indicate that erythrocytes are aggregated by electrolytes as electronegative colloids at pH concentrations below the isoelectric point of hemoglobin and by direct union with metallic salts under the same conditions that function for proteins in general. Gelatin prevents the colloidal aggregation, or agglutination, but not that due to direct chemical union with its accompanying precipitation and coagulation of the erythrocytic contents. The plasma proteins are precipitated at their specific isoelectric points. This precipitation alone is insufficient to cause clumping in the pipettes. If the cells aggregate, on the other hand, because of their own forces and irrespective of the events occurring in the proteins in which they are suspended, then the precipitated proteins serve to qualify and further facilitate clumping by becoming balled with the cells and in turn entangling more cells. Balling and clumping are exaggerated and emphasized with the gentle and regular motion of the rotor, but are inherent in the reactions of blood with bichloride. When the cells are protected from aggregation the rotor is unable to produce clumping. As Ch'u and Forkner<sup>4</sup> found with hand shaking, these same features are naturally present in other methods of shaking. It is merely that most methods of shaking offer enough violence and shift of direction that the colloidal aggregates are prevented from holding, and in that case the precipitated proteins do not find a nucleus for collection and a reciprocal entangling of more cells. Clumping therefore is not in evidence with such methods of shaking, but the underlying potentialities are present nonetheless, and the dangers of clumping are always present. Ch'u and Forkner<sup>4</sup> found this to be particularly true in certain pathologic conditions.

The experimental findings are significant from the standpoint of various clinical and biologic studies other than blood counts, and a brief survey of certain of these seems indicated.

The fact that modifications in pH in the presence of tissue fixatives can effect such marked changes in erythrocytes as lysis or agglutination, respectively, emphasizes anew the importance which Petunkévitch<sup>6</sup> ascribes to pH in the fixation and staining of tissues. The misconceptions that may occur because of failure to evaluate this factor are discussed in many texts on histologic technique. The fact that differential precipitation of the different elements of the blood exposed to the same fixative may occur simply under the influence of different hydrogen-ion concentrations indicates possibilities for differential analysis of the finer cytology of tissues. Obviously the differences in the way

tissues react to the same stain are due not only to the simple acid base relationships upon the stains or between the stains and the tissues but also are determined by the physical state of each element in the tissues that, in turn, having been determined by the pH of the fixative in relation to the elements in question. These possibilities are very beautifully illustrated in the differential staining of anatomic cross sections which Robin<sup>8</sup> has obtained with aniline blue used at different hydrogen ion concentrations.

The lysis of erythrocytes that has been shown to occur in weak solutions of bichloride and of the other fixatives employed is probably due either to solution of cell membranes or to some sort of union of the membranes with the fixatives involved in such a manner as to weaken the membranes and allow extrusion of the contents before coagulation or fixation of the latter can occur. In the case of the weak solutions of bichloride the union is probably with the lecithin of the capsule while the concentration of bichloride or the pH or both are insufficient to permit coagulation of the proteins. In stronger concentration the amount of bichloride is sufficient to coagulate or fix the proteins of the cells even as it unites with the lecithin of the capsule. The action of gelatin as a protective colloid applied to the surface of the erythrocytes probably explains its effect in preventing lysis just as it prevents agglutination.

The capacities of dilute solutions of fixative agents to cause lysis and especially of very dilute solutions containing mercury at hydrogen ion concentrations equal to those which obtain in blood and tissues place significance upon the possibility of equivalent action in local or generalized areas subjected to therapeutic agents of similar nature.

It is significant that the hydrogen ion concentration of the blood is such as to favor precipitation of plasma proteins and agglutination of erythrocytes in the presence of electropositive metals and colloids especially those represented by a heavy metal like mercury. The importance of this fact in therapeutic and diagnostic procedures employing such solutions is obvious. When such solutions are used for parenteral therapy for instance the events when they meet body fluids must often mark the difference between smooth and stormy reactions. And in such diagnostic procedures as the use of Hyman's solution<sup>9, 10</sup> or other solutions containing bichloride<sup>11, 12</sup> in studies of liver function, the ambiguities might well be lessened by regulation of the pH. The recent work of Kunkel<sup>11</sup> with solutions of copper or zinc for a similar purpose certainly shows the importance of pH in the results and interpretations of such tests.

The dangers of unpredicted agglutinations are lessened in the presence of the correct protective colloids for the situation at hand but the protection from such colloids may be negated by elevation or depression of the pH of the environment to that at which the colloid itself is precipitated by the reagent.

The fact that the gentle rotation of blood in the fixed planes set by the Bryan Garrey rotor brings out the inherent tendency for precipitation and clumping in red blood counts suggests that similar motion might prove useful in biologic and diagnostic procedures that require detection of the phenomena of precipitation or agglutination. In certain respects the method achieves the

same results as are sought at times by vibratory mechanisms.<sup>14</sup> In the present studies the method actually allowed detection of the phenomenon of aggregation when it was masked by the more violent vibration of a shaker or even by hand shaking.

The risks inherent in the use of soft glass containers are once again illustrated in the present studies. Storage of Hayem's or Jørgensen's solution in soft glass containers quickly resulted in massive clumping with whole blood under conditions in which this phenomenon did not occur if the diluents were stored in Pyrex containers. These effects were caused not only by changes in pH due to reaction with soft glass, but also by changes in the content of electrolytes.<sup>5</sup> Since the findings and the interpretation of them are obviously tremendously influenced by the pH and the electrolytes of the test solutions in diagnostic procedures based on precipitative or agglutinative phenomena by means of solutions containing heavy metals, the dangers of change in these factors by improper storage are very real. The reactions between the metal and the biologic substance in question may be encouraged, suppressed, or confused by reactions of other substances, depending upon these factors.

The power of erythrocytes to attract and carry colloidal substances, while recognized, is given far too little consideration in biologic studies. The action of gelatin, and to a lesser extent, that of the plasma proteins in the prevention of aggregation of erythrocytes in these studies illustrates the surface force of the erythrocytes, even as it indicates the colloidal activity of these proteins. The studies of Bloom<sup>16, 17</sup> indicate that after fats have been absorbed from the gut they first are attracted from the plasma to the cells of the blood before chemical changes can take place within the cells. Pennell<sup>18</sup> recognizes surface attraction between erythrocytes and platelets in his concept of a causal relationship between erythrocytes and hemophilia.

#### CONCLUSIONS

The factors involved in the clumping of blood in red counts made by the aid of mechanical rotation are inherent in the interactions of Hayem's solution with plasma and cells and are merely emphasized by rotation. The capacity of rotors to demonstrate the phenomena of precipitation and aggregation and/or agglutination in blood counts was found useful in elucidation of the factors involved in these phenomena.

The clumping is due to the reactions of mercuric bichloride with the cells and the plasma as a tissue fixative. These reactions are regulated by (1) the concentrations of bichloride, (2) the relationship of the pH of the diluent to the isoelectric points of the erythrocytic contents and of the plasma proteins, and (3) the colloidal activities of intact erythrocytes.

When dilute enough, bichloride may cause lysis. When concentrated enough it causes colloidal aggregation and/or agglutination of the erythrocytes and precipitation of proteins, depending upon the pH. Addition of gelatin to the diluent protects the cells from lysis and aggregation at hydrogen ion concentrations below pH 7.0, but does not affect the precipitation of proteins.



Aggregation of the erythrocytes and precipitation of the proteins under the action of bichloride occur independently of each other. Aggregation alone may simulate clumping. Precipitation of the plasma proteins alone does not cause clumping. When aggregation accompanies precipitation characteristic clumping occurs.

The findings are discussed from the standpoint of their significance in relation to problems other than blood counts. They bear upon the phenomena of lysis, precipitation, and agglutination upon diagnostic and therapeutic measures which involve colloidal or precipitative reactions and upon the differential responses of tissues to fixation and staining.

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# MEASUREMENT OF THE ELECTRIC RESISTANCE OF HUMAN BLOOD, USE IN COAGULATION STUDIES AND CELL VOLUME DETERMINATIONS

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THE measurement of electric resistance and conductivity is a universally recognized physical-chemical procedure which has had wide use in biologic investigations. It has been successfully used in the study of cell permeability, muscle physiology, and so forth, and attempts have been made to measure electric resistance changes during the blood coagulation process. Most of the results obtained have indicated that there is no blood resistance change during coagulation. A review of the literature reveals that most of the work done on this subject was published before 1926 and centers around resistance or conductivity as related to cell volume and coagulation. Stewart and others<sup>1-5</sup> have shown that the resistance of blood is directly proportional to the cell volume. Blood may be thought of as a suspension of cells, which are very poor conductors, and plasma, whose conductivity depends upon the concentration of electrolytes, particularly sodium chloride<sup>6</sup>. Sigman and co-workers<sup>7</sup> measured the resistance of beef blood flowing through a tube. They reported that the resistance decreases with increasing flow velocity. Other workers<sup>8-10</sup> have employed resistance measurements in studies of hemolytic and osmotic behavior of the red cell. Resistance of packed red cells, normally thirty to forty times the resistance of plasma, is reduced to a value comparable to that of plasma by saponin hemolysis.

Most authors were unable to demonstrate reproducible electric resistance changes during the complex process of blood clotting<sup>11-14</sup>. In the only recent report, Giaff and co-workers<sup>15</sup> say that there are irregular resistance changes during clotting, not related to clotting time, and that the resistance of heparinized blood measured in vitro steadily increases in the first half hour after obtaining the blood sample.

In the present paper a reliable and practical experimental method is described for the measurement of electric resistance of blood and other fluids. Observations are presented to show that this method enables one to make reproducible resistance versus time measurements on whole blood and that the varying observations of previous investigators may have been due to inadequate technique. These observations apply to changes in blood resistance during coagulation and to the relation between blood resistance and cell volume fraction.

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## METHOD

The application of the classic method of Kohlrausch and Holborn<sup>16</sup> and its elaboration worked out by Washburn and others has been widely accepted for the measurement of the electric resistance of colloid systems and suspensions. Taylor and Acree,<sup>1</sup> Washburn and Weiland,<sup>18, 19</sup> and others<sup>20, 21</sup> have given excellent discussions of the theoretic principles. It is beyond the scope of this paper to discuss technical details other than those applying especially to the determination of the resistance of blood. Most of the experimental arrangements for blood resistance measurements reported in the literature appear to have been unduly complicated and time consuming and intended for a limited number of measurements. We believe we have designed a setup which can be used to study the clotting of blood, permitting rapid successive resistance measurements. The method has high reliability of single measurements and it could be adapted to measurement of other biologic materials.

Fig. 1 shows the measuring tube and electrodes. The tube made of Pyrex, was 12 mm in diameter and could be held in any test tube rack. The electrodes, consisting of lightly platinized\* platinum plates 0.01 inch in thickness  $\frac{1}{4}$  sq cm in area, and  $\frac{1}{2}$  cm apart, were designed to be large enough to avoid the errors arising in the use of wire point and small electrodes and yet to require only about 15 cc of blood or other fluid. Platinized electrodes eliminated the necessity of extrapolating the measurements to infinite frequency as required in the case of unplatinized (bright) electrodes, and they gave a sharper end point of impedance balance. Also measurements during clotting made with unplatinized electrodes often showed discrepancies which could be explained by imperfect adherence of the clot to the electrodes. The electrodes were cleaned satisfactorily by connecting them as cathode in 20 per cent sodium hydroxide and applying 3 volts for two minutes. The adherent fibrinous material loosened by this process readily washed off with hot distilled water.

The position of the electrodes in relation to the measuring tube and blood clot determines the result of measurement to some extent. In the geometry used in the present experiments the electrodes were entirely contained within the clot and they remained in the clot during retraction (see Fig. 2). This enabled us to study the change of resistance during clot retraction for a considerable length of time. Unfortunately, some previous investigators did not describe their electrode arrangements. It was found by us that if electrodes are placed too distantly or in an inconvenient way the clot may break off during the process allowing plasma to interfere with the clot resistance measurement. The results obtained with such latter electrode arrangements are irreproducible.

An audio frequency oscillator capable of producing an alternating current frequency from 50 to 200,000 cycles per second was used to generate power. Since measurements with the platinized electrodes showed negligible variation with frequency 1,000 cycles/sec. was used through the experiments.

The impedance bridge† provided an accuracy adequate for our purposes with a probable error of less than 1 per cent and a maximum error of 2 per cent. The amplitude of the applied voltage was 0.25 volts root mean square. Capacitance of the measuring cell circuit was negligible and compensation did not alter the readings. Our parallel control observations showed that the behavior of clotting blood was not changed beyond the range of experimental error by the current used in the measurements.

Instead of using the conventional telephone bridge balance indicator, an oscilloscope‡ was used. The horizontal sweep was connected to a 60 cycle saw tooth power supply. The

\*Electrodes were platinized with Lummer-Laurbaum solution. 0.3 Gm. platinum chloride 0.002 Gm. lead acetate dissolved in 10 cc. of distilled water. Six volts were applied, each electrode connected as a cathode for thirty seconds. Electrodes were cleaned by N sulfuric acid, connected as anode 3 volts for 1 and one half to 2 minutes. Electrodes had to be replatinized when the platinum black surface became inefficient.

†Hewlett Packard Palo Alto Calif. model 60 C.

‡General Radio Co. Cambridge Mass. type 650 A.

§Cathode ray oscillograph Type 294 Du Mont Laboratories Passiac N. J.

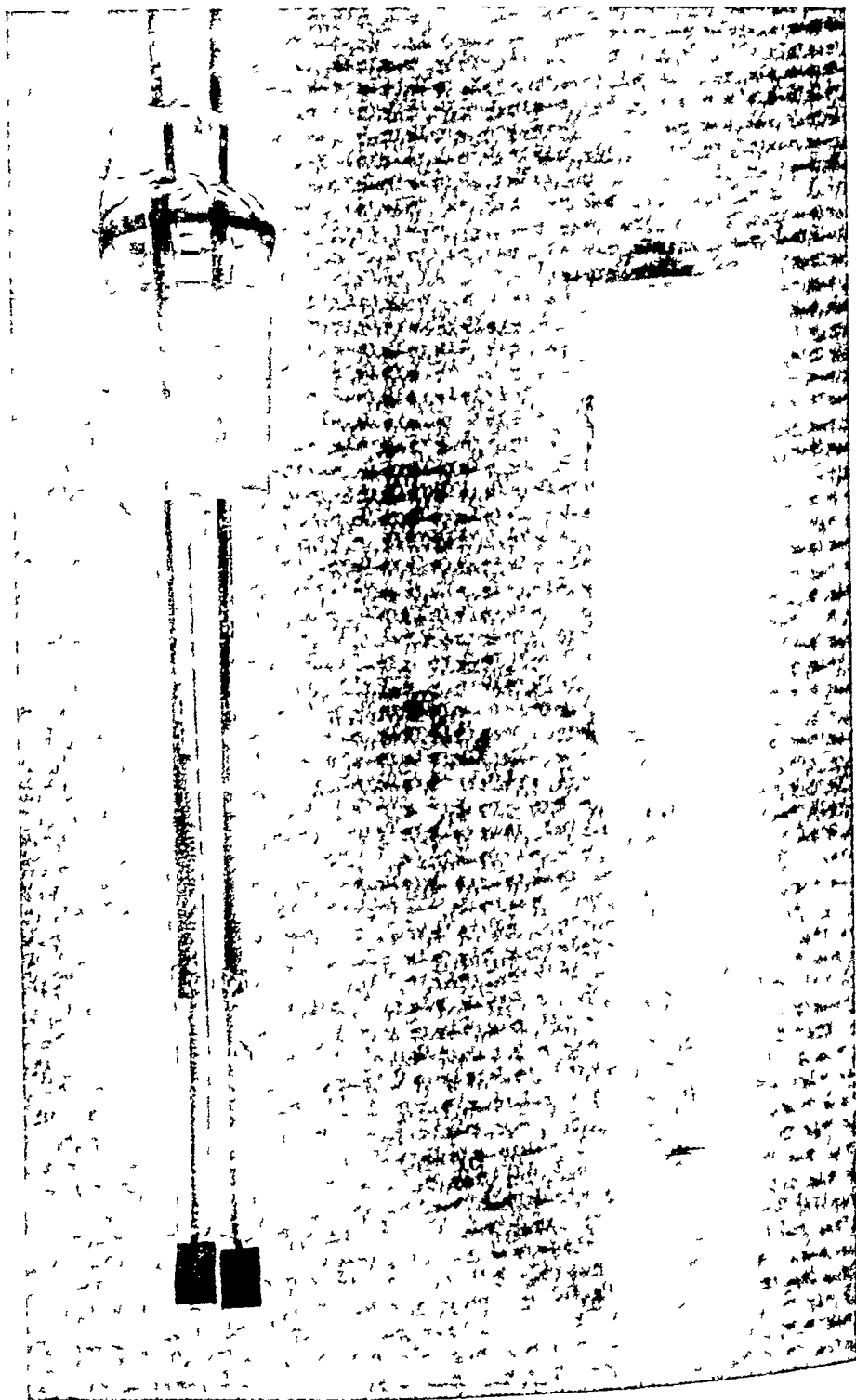


Fig 1—Measuring tube and electrodes

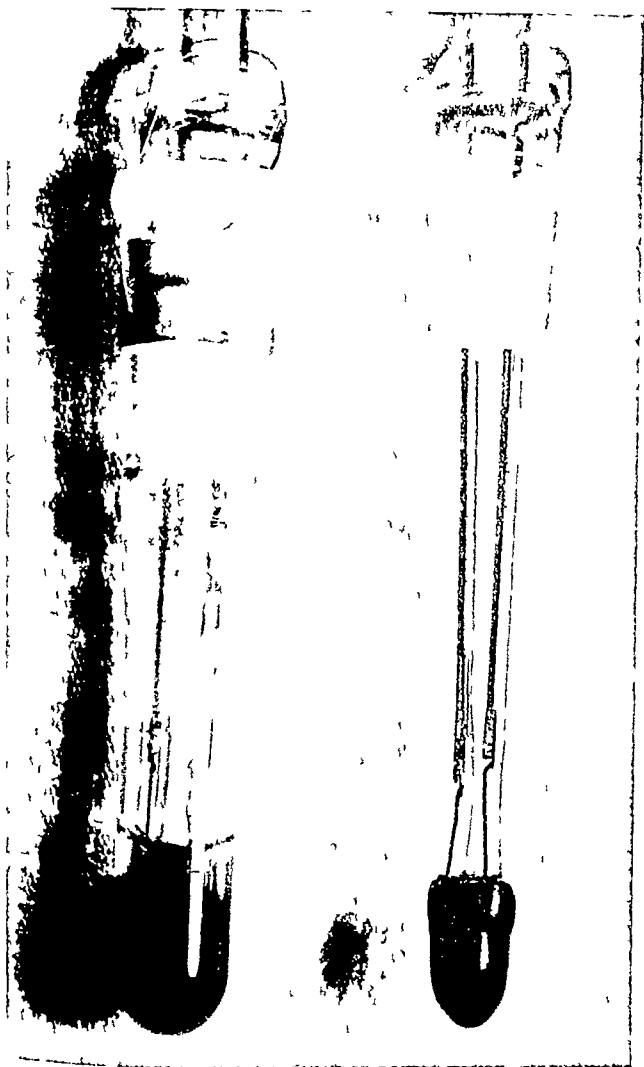


Fig — Appearance of retracted clot in situ in the measuring tube and its adherence to the electrodes on their removal from the tube

vertical sweep showed the amplified 1,000 cycle A C voltage appearing between the unbalanced junctions of the impedance bridge. At balance, the vertical amplitude reached a well defined minimum.

By means of a selector switch and parallel circuits, six different samples could be studied at one time. All determinations were made in a constant temperature water bath set at 37° C (See Fig 3). An increase in temperature of 1° C produced an approximate 2.3 per cent decrease in resistance. The conductivity cell constant was measured with 0.1 KCl and all measurements were converted to specific resistance in ohm centimeter units by the relation

$$\text{Specific resistance at } 37^{\circ} \text{ C} = \frac{\text{Measured resistance of blood}}{0.0158 \times \text{Measured resistance of } 0.1 \text{ N KCl}}$$

where the factor 0.0158 is the specific conductance of 0.1 N KCl at 37° C

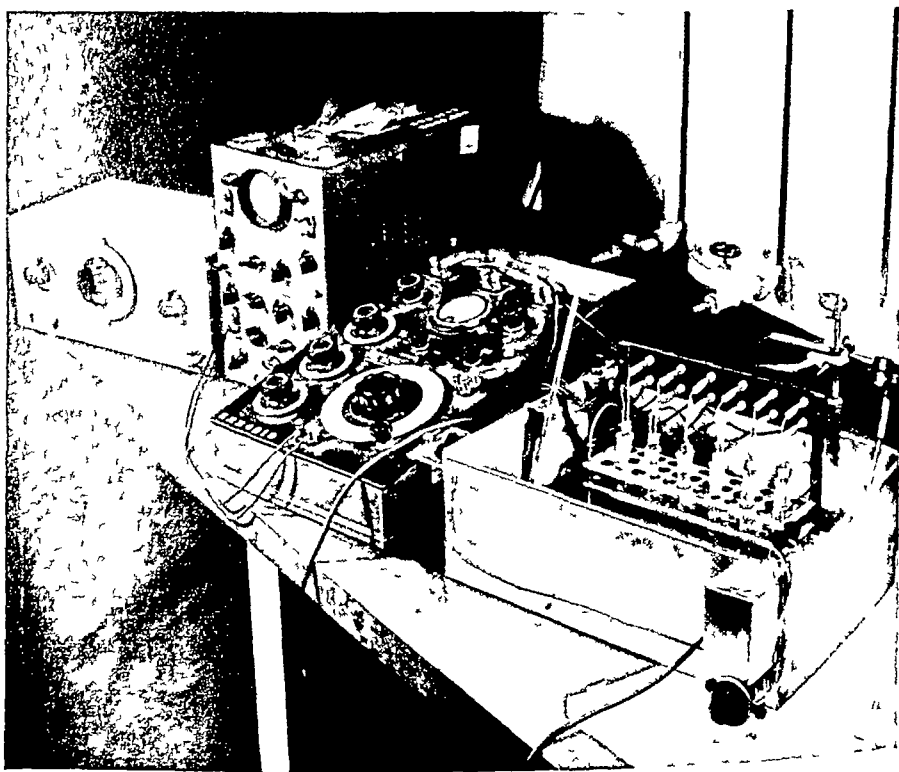


Fig 3—Left to right, audio frequency oscillator, oscilloscope, impedance bridge, water bath and stand with six measuring tubes connected for making measurements.

#### OBSERVATIONS

*Resistance Changes During Blood Coagulation*—15 cc of blood drawn with a dry syringe were carefully placed in the measuring tube, the electrodes were inserted, and measurements were made at frequent intervals for forty minutes. Parallel clotting time determinations were performed by a modified Lee-White method (½ cc of blood, two tubes 10 mm in diameter). The results are shown in the two curves in Fig 4, with time plotted against specific resistance in ohm-centimeter units. The lower curve represents the

average of measurements on sixteen blood normal specimens while the upper one is composed of the average of fourteen polycythemic blood samples. All blood samples showed clot retraction. The curves for two individuals together with the clotting times are shown in Fig 5. For the first few minutes the resistance value was constant at a level indicative of the cell volume per cent. Then at the time clotting occurred, the resistance started to increase. This increase, which is related to clot retraction has been found to continue for at least seven or eight hours. Each of the determinations followed this pattern, with variations occurring in the magnitude of resistance point of beginning resistance increase, and slope of the curve. The higher value for the one minute determination could be explained by the temperature adjustment and possibly by insufficient time for thorough wetting of the electrodes. The slight drop in temperature of the blood while in the syringe would increase the resistance 2.5 per cent for each degree centigrade.

Definite substantiation of clotting as the factor responsible for the time resistance curve pattern described was obtained by observations on blood rendered incoagulable by the *in vitro* addition of heparin (0.1 mg) or oxalate (18 mg ammonium and 12 mg potassium oxalate). The unclotted blood gave no significant change in resistance over a one hour period as seen in Fig 6 which also shows the same blood, without any addition of an anticoagulant giving the typical clotting pattern. Very slight variations in resistance were the probable effect of red cell sedimentation to produce changing cell

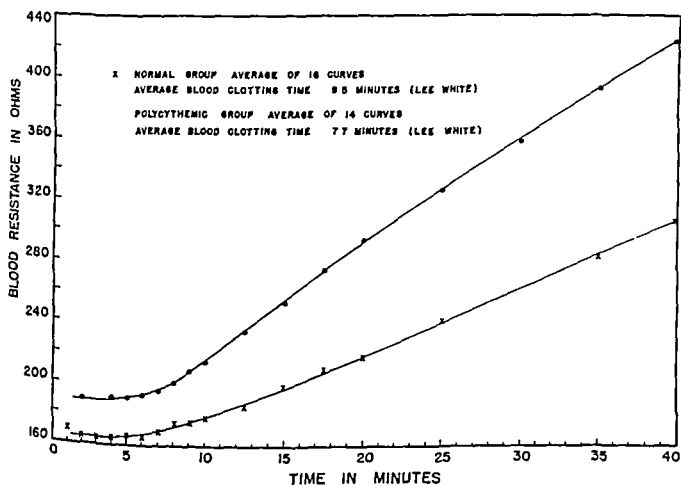


Fig 4—Curves of the average blood resistance during clotting for sixteen normal and fourteen polycythemic subjects.

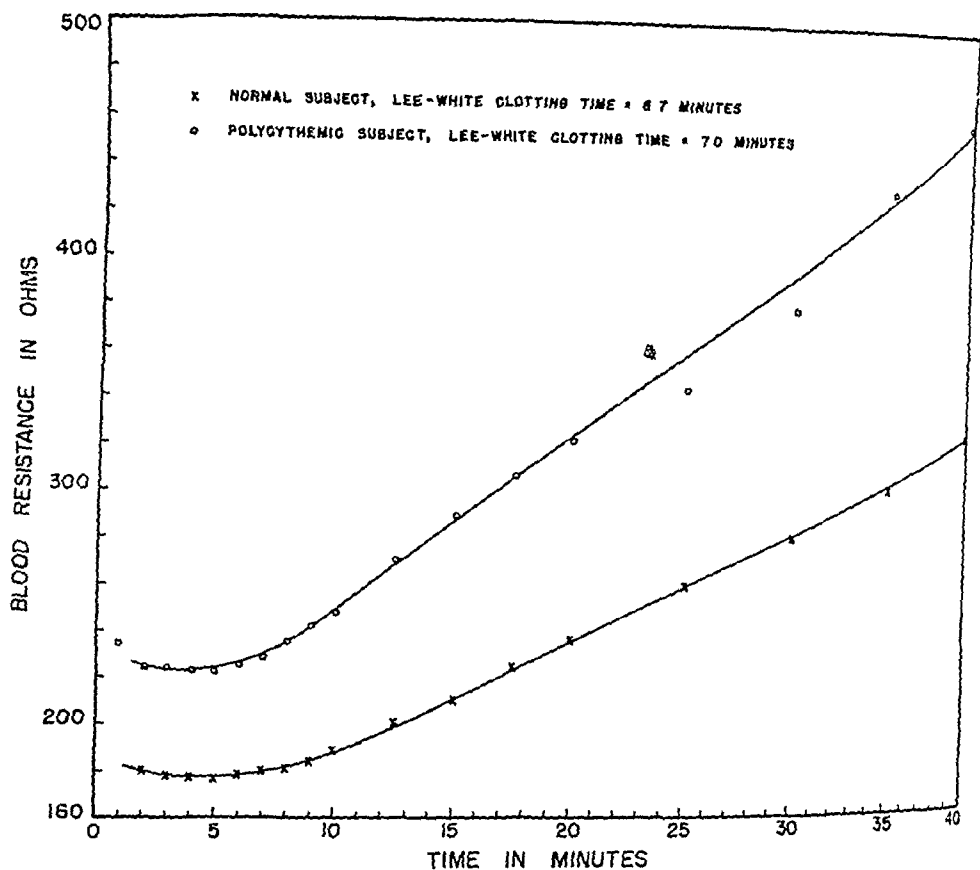


Fig 5—Blood resistance curves during clotting for a normal subject and a polycythemic subject.

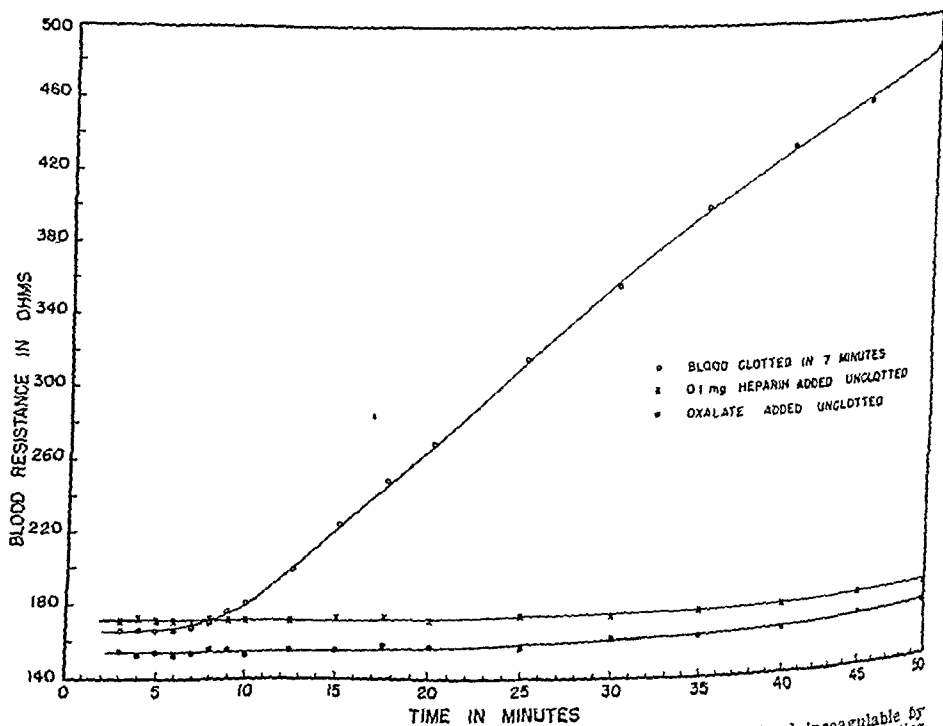


Fig 6—Resistance measurements showing the effect of rendering blood incoagulable by the addition of anticoagulants such as heparin and oxalate. The changes during clotting for the blood of the same subject are shown for comparison.



concentrations around the electrodes. It was found that shaking or stirring the unclotted blood increased the resistance, which dropped in one or two minutes to a constant value about 6 per cent lower as the cells settled.

*Resistance and the Cell Volume Fraction of Blood\**—The electric resistance of plasma is much lower than that of blood cells alone. Whole blood resistance is determined by the intrinsic resistance of plasma and cells and by the relative amount of cells and plasma present in the blood. It also has been shown that the shape of the red blood cells influences whole blood resistance. Ponder<sup>4</sup> has presented a formula for calculating the cell volume fraction of a sample of blood from the measurement of the resistance of both the unclotted blood and its plasma separated by centrifugation.

$$\text{Cell volume fraction} = \frac{\phi - \frac{1}{X}}{\phi + \frac{1}{X}}$$

Where  $\phi = \frac{\text{resistance of blood}}{\text{resistance of plasma}}$  and  $X$  is the form factor dependent on the shape of the cells, assumed to equal 1.10 for red cells in plasma. Also, cell volume per cent = cell volume fraction  $\times 100$ .

In the present study the cell volume per cent was determined by both electrical resistance measurements and centrifugation (Wintrobe tube heparinized blood, 2,500 revolutions per minute for one half hour reading represent red cell volume) on aliquot samples of venous blood. Plasma values averaged 63.2 ohm centimeters with a range of 61.0 to 66.8 ohm centimeters. The blood resistance, taken as the average value of the three four and five minute readings of the time resistance curve ranged from 131.2 to 230.9 ohm centimeters. A comparison of the results obtained by the two methods revealed that the resistance determinations were 7.7 per cent lower than the centrifuge values in normal subjects and 7.8 per cent lower in patients with polycythemia vera for average values (see Table I). In three leucemic blood specimens with an average white count of 96,200 the resistance method compared 5.7 per cent higher than the centrifuge method. This may be explained by the fact that while only the red cell volume was recorded for the centrifuge cell volume, the resistance measurement accounted for the  $H_2O$ , poorly conducting white cell volume as well as the red cell volume. For normal or only slightly elevated white counts the white cell resistance factor was very small.

The cell volume per cent can readily be determined from resistance measurements by taking the ratio of blood to plasma resistance ( $\phi$ ) and re-

Since the precise meanings of the terms cell volume and hematocrit are at the present time confused we deem it advisable to clarify certain terms used in this paper. Hematocrit is used in its strict sense to refer to the volume of packed red cells expressed as a per cent, obtained by centrifugation of 1 c.c. of blood in a tube 100 mm long. The term cell volume has been used to designate the proportion of cells by volume in a given sample of blood. The total cell volume of an animal or volume of an individual cell. In this paper the term cell volume fraction is used to indicate the proportion of cells by volume in venous blood irrespective of the method used in its determination. The volume per cent of cells in the blood sample is termed cell volume per cent irrespective of its method of determination. Since the volume of white cells unless abnormally increased as in leucemia, is very small the cell volume per cent approximates the red cell volume.

fering to a curve of the cell volume per cent values calculated from Ponder's equation plotted against  $\phi$  as in Fig 7. The values obtained by the centrifuge method are plotted against  $\phi$  for comparison. Table II lists some pertinent data on subjects used in these measurements.

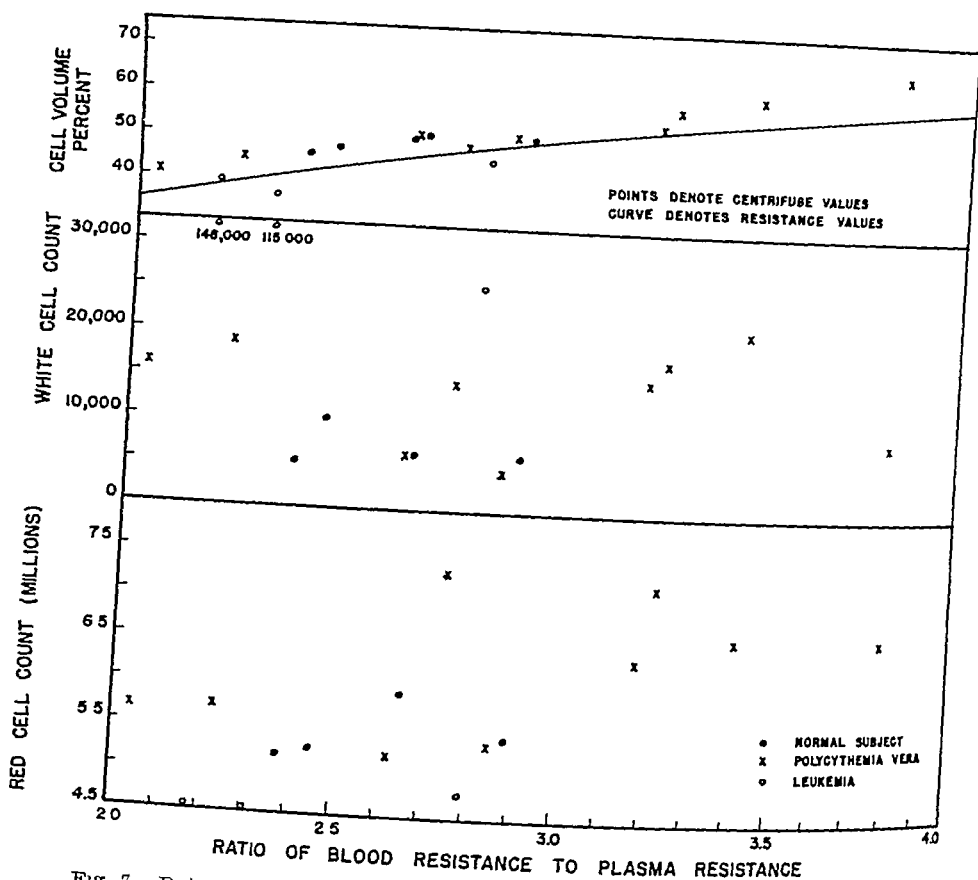


Fig 7—Ratio of blood to plasma resistance ( $\phi$ ) plotted against cell volume per cent, white count and red count for seventeen subjects. The absence of a simple relation between blood resistance and white and red counts is shown. There is a direct relation of resistance to cell volume (chiefly red cell). The curve represents cell volume per cent values obtained by calculation from Ponder's formula using the ratio of blood resistance to plasma resistance ( $\phi$ ).

In order to obtain a wider range, heparinized blood was diluted and concentrated by varying the proportion of cells to plasma, and cell volumes were determined by the two methods on aliquot samples. The blood resistance measurements were obtained immediately after shaking, before settling had occurred. Results, shown in Table III, indicated that the resistance determinations were 61 per cent lower for the normal group, 81 per cent lower for polycythemic groups, and 20 per cent lower for the leucemic group. The combined normal and polycythemic groups gave a value of 73 per cent.

The level of blood resistance showed no direct relation to hemoglobin, red cell count, white cell count, or platelet count. Fig 7 shows the red and white counts plotted against the blood-plasma resistance ratio ( $\phi$ ).

TABLE I CELL VOLUME PER CENT OF VENOUS BLOOD DETERMINED BY ELECTRIC RESISTANCE AND CENTRIFUGE METHODS

GROUP	NUMBER OF SAMPLES	AVERAGE CELL VOLUME PER CENT		CELL VOLUME PER CENT DIFFERENCE	AVERAGE RED COUNT	AVERAGE WHITE COUNT
		RESISTANCE	CENTRIFUGE			
Normal	5	45.5	49.2	7.7	5,490,000	7,100
Polycythemic	3	48.8	52.9	7.8	6,230,000	13,630
Leucemic	3	42.4	40.1	+5.7	4,570,000	96,200

Cell volume per cent difference represents the difference between the resistance and centrifuge cell volumes expressed as per cent of centrifuge value

TABLE II BLOOD AND PLASMA RESISTANCE DATA FOR NORMAL POLYCYTHEMIC AND LEUCEMIC SUBJECTS

SUBJECT	SPECIFIC RESISTANCE (OHM CM)		RES. BLOOD	CELL VOLUME PER CENT		RED CELL	HEMOGLOBIN	WHITE CELL COUNT	PLATELETS
	BLOOD	PLASMA	RES. PLASMA	RESISTANCE   CENTRIFUGE		COUNT	BIN		
			Φ						
Normal									
J. M.	141.6	62.1	2.38	41.8	46	5.4	15.0	5500	—
A. C.	163.8	66.8	2.45	43.2	48	5.9	14.5	10100	320000
L. D.	160.7	61.0	2.63	46.0	50	—	—	—	—
B. W.	173.6	65.6	2.65	46.3	51	5.4	14.0	6300	—
L. D.	166.3	61.0	2.89	49.7	51	5.48	13.0	6600	240000
Polycythemia									
A. F.	151.2	64.3	2.04	35.2	41	5.68	11.0	15750	130000
A. F.	143.5	64.4	2.23	39.0	45	5.72	10.6	18000	680000
W. M.	169.1	64.4	2.63	46.0	51	5.20	13.0	6100	400000
W. P.	180.0	65.6	2.74	47.7	48	7.30	12.0	14500	340000
A. I.	174.7	61.3	2.85	49.2	51	5.6	13.5	4700	200000
M. P.	205.1	64.5	3.18	53.4	54	6.55	14.8	15400	400000
A. P.	199.8	62.0	3.22	53.8	58	7.20	15.5	17500	350000
I. P.	3.5	65.5	3.41	55.8	61	6.70	15.6	21200	350000
M. R.	2.09	61.0	3.78	59.2	61	6.49	15.0	8150	370000
Chronic Lymphatic Leucemia									
M. R.	137.8	63.2	2.18	38.2	39	4.45	11.6	145000	210000
M. R.	143.0	62.0	2.31	40.6	36	4.46	11.5	115000	250000
P. B.	170.3	61.0	2.79	48.3	43.3	4.81	12.0	25700	140000

TABLE III CELL VOLUME PER CENT OF VARIOUS DILUTIONS AND CONCENTRATIONS OF HEPARINIZED VENOUS BLOOD

SUBJECT	NUMBER OF SUBJECTS	NUMBER OF OBSERVATIONS	AVERAGE CELL VOLUME PER CENT		CELL VOLUME PER CENT DIFFERENCE*
			RESISTANCE	CENTRIFUGE	
Normal	5	10	53.5	55.0	-6.1
Polycythemic	9	16	50.9	55.4	-8.1
Leucemic	3	4	33.9	34.6	-2.0
Normal and Polycythemic	14	26	51.9	56.0	-4.3

\* See footnote to Table I

## DISCUSSION

Our results show blood resistance measurement to be of value in the study of blood coagulation, both as a method for the detection of the clotting time and as a means for obtaining the quantitative measurement of the rate of clot retraction. The advantage in the use of electric resistance lies in the fact that a dynamic process such as blood coagulation may be studied under controlled conditions without disturbing the process by the making of measurements.

Emphasis must be placed upon the importance of the geometric orientation of the electrodes to the clotting blood and retracting clot in the evaluation of observations and data. We located our electrodes at the central part of the blood and within the retracting clot. As soon as the blood has clotted, clot retraction begins by the contraction of the fibrin network which pulls the large elements or cells together into a dense mass, thus displacing the serum to the periphery. This process produces increases in resistance measurements because it simultaneously increases the concentration of poorly conducting cells and decreases the concentration of serum, a good conductor, between and around the electrodes. On this basis it is possible to relate our observed changes in resistance during clotting, as graphically demonstrated by the time resistance curve, to blood coagulation events. Prior to clot formation there is no significant change in resistance. The clotting time and start of clot retraction are marked by the first increase in resistance. Thus the clotting time may be determined with the elimination of motion, the source of considerable variation and inconsistency in most methods in common use.

The subsequent increases in resistance result from retraction of the clot. Therefore the slope of the rising portion of the time-resistance curve may be assumed to correspond to the rate of clot retraction and to serve as a quantitative measure of this process. Since there has been no method previously available for a comparable quantitative study of clot retraction, such measurements may serve to detect significant differences in and variations of this process in disease. The method also may provide a means to determine the effect of chemical and therapeutic agents upon clot retraction.

Our results differ from those reported by Graff and co-workers<sup>15</sup> who obtained an increase in resistance of normal blood during the first ten minutes unrelated to clotting time, while heparinized blood gave a rapid progressive increase in resistance for twenty to thirty minutes. The description of the method given by these authors is not complete enough to allow us to compare their data with ours. Certain important features of their method such as the use of 60 cycles A C and applied voltage of 5 volts root mean square appear to be at variance with accepted procedures which have been thoroughly worked out for conductivity measurements.

It is of investigative interest to compare the cell volume fraction calculated from resistance measurements with the centrifuge hematocrit. It has been known for some time<sup>3</sup> that hematocrit values obtained by separation of cells from plasma in the centrifuge do not give the true cell volume fraction. This

would be true only if the centrifuge would pack the cells so closely that all intercellular space and plasma would be excluded from among the cells. For example, Miller<sup>20</sup> and Kennedy and Millikan<sup>2</sup> demonstrated that in order to achieve such perfect separation, centrifugal force of a magnitude that would destroy the cells themselves would be required. There are a number of papers available which compare various methods for the determination of the cell volume fraction with the centrifuge method. Kennedy and Millikan<sup>2</sup> and Shohl and Hunter<sup>28</sup> reported values obtained by using dye dilution of plasma 10 to 12 and 4.5 per cent, respectively, below the centrifuge hematocrit. Chapin and Ross<sup>29</sup> found the cell volume fraction determined by dye dilution (T 1824), protein dilution, and the use of red cells tagged with radioactive iron to be an average of 8.5 per cent lower than the cell volume fraction obtained by centrifugation. Ponder and Saslow<sup>3</sup> calculated the cell volume fraction in whole blood from direct measurement of the dimensions of the individual red cell and then number. This calculated value was in close agreement with the dye dilution determination of the cell volume fraction of the same blood. Stewart<sup>3</sup> used the method of conductivity and also obtained values below the centrifuge hematocrit (5 to 6 per cent). The results presented in this paper indicate that the cell volume fraction determined by electric resistance measurements is 7.7 per cent lower than values obtained by centrifugation. Thus the dye dilution, protein dilution, tagged red cell, direct measurement, and electric resistance methods agree very well in the determination of the fractional volume of red cells in blood, while the value obtained in the centrifuge hematocrit is consistently high because of plasma trapped between the packed red cells.

## SUMMARY

A reliable rapid method for the measurement of electric resistance of small amounts of blood or similar material is presented.

A pattern of change in the resistance of blood during coagulation is described. These resistance changes make possible the determination of the clotting time with elimination of inconsistencies caused by motion and offer a quantitative means for the study of clot retraction. In view of the fact that no method has been available previously for a comparable quantitative study of clot retraction, electric resistance measurements may serve to detect significant variations of this process in disease beyond our present knowledge.

By means of the ratio of blood resistance to plasma resistance the cell volume fraction of a sample of blood may be calculated. Cell volumes determined by resistance measurements were found to average 7.7 per cent lower than the hematocrit as determined by centrifugation.

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# THE EFFECT OF CHOLINE, METHIONINE, AND LOW FAT DIET ON THE LIFE EXPECTANCY OF PATIENTS WITH CIRRHOSIS OF THE LIVER

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IN A previous communication from this clinic a regimen for the treatment of cirrhosis of the liver was described<sup>1</sup>. This regimen was based upon two assumptions first, that fatty changes in the liver ordinarily precede the more characteristic anatomic lesions of cirrhosis second that fatty changes in the liver may be dietary in origin especially in the alcoholic patient and hence might be comparable to the changes observed in experimental animals placed on deficient diets. The clinical course of several patients was described. The results attributed to the therapy were striking in each instance. It was pointed out, however, that not all patients had responded so dramatically and that spontaneous remissions have long been known to occur in the absence of any specific therapy. Interpretation of the efficacy of the therapeutic regimen therefore had to await the study of an increased number of patients over a longer period of time.

## METHOD OF STUDY

The present report summarizes the clinical experiences of the past five years during which time some 224 patients with cirrhosis of the liver were studied. The fate of these patients is compared with that of a similar control group of patients treated in the same hospital under similar circumstances prior to the use of the present therapeutic regimen.

The therapeutic regimen used in the treatment of the present series of patients is summarized in Table I.

**Diet**—The protein intake was maintained as near 100 Gm per day as possible so that presumably adequate supplies of methionine and cystine as well as other essential amino acids were provided. The fat intake was limited to approximately 50 Gm per day. It seemed unreasonable to burden further a fatty liver with additional supplies of fat since it was assumed and later demonstrated experimentally<sup>4</sup> that under some circumstances at least much of the liver fat was exogenous in origin. Animal fat was particularly avoided since cholesterol is known to be one of the most potent stimulants to fat infiltration. Such fat infiltration is reported by some observers to be exceedingly resistant to lipotropic agents<sup>2</sup>. Carbohydrate was given ad libitum (400 to 600 Gm) and in sufficient quantities to spare dietary protein for more essential purposes. The total caloric value of the diet was approximately 3,000 calories.

**Drugs**—More specific attempts were made to supply choline and methionine. At first 1 Gm of choline chloride was given daily in divided doses. Since it

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TABLE I THERAPEUTIC REGIMEN

Diet	
Protein	90 100 Gm +
Fat (chiefly vegetable)	50 Gm
Carbohydrate	Adequate to supply caloric needs
Drugs	
Choline hydrochloride	1 8 Gm
Methionine (1 qt skimmed milk)	0 9 Gm +
Vitamins	
A	10,000 U S P units
D	800 U S P units
Abstinence from alcohol	

had been emphasized by some observers<sup>3, 5</sup> that large doses of choline given over a prolonged period of time were required once liver damage was established, the dosage was increased cautiously on the basis of the patient's tolerance. Few were able to take more than 6 Gm, and none more than 8 Gm without experiencing gastrointestinal symptoms which were thought to be referable to the choline chloride. Most patients took and tolerated 3 to 5 Gm daily very well.

Since pure methionine was obtainable with difficulty and at a considerable cost at the outset of these observations, milk was used as a source thereof. All patients consumed at least one quart of milk (skimmed to avoid unnecessary animal fat) daily. This is equivalent to approximately 0 9 Gm of methionine.

*Vitamins*—Supplements of the fat-soluble vitamins A (10,000 U S P units) and D (800 U S P units) were given in order to compensate for the restrictions of dietary fat. Supplements of the B complex were not used so as not to complicate further the evaluation of the therapeutic program.

*Alcohol*—Alcohol consumption was prohibited, not because of any fear of the alcohol per se, but rather because the therapeutic regimen usually was followed inadequately if the patient continued to imbibe. Whenever it was apparent that cooperation in this respect was not probable, the patient was kept in the hospital for long periods of time (six to eight months) and often on repeated occasions. No similar effort was made to govern the alcohol intake of the control group. While it is difficult to evaluate the relative extent to which the members of the two groups heeded the warning to abstain, it is very probable that far better control was maintained in the experimental group.

#### CASE MATERIAL

Those patients admitted to the St. Louis City Hospital during the eleven year period beginning April 1, 1935, and upon whom the diagnosis of cirrhosis of the liver was made are the basis of the present report. The patients were divided into two groups: the experimental and control groups. The former included those patients with cirrhosis who were admitted to the hospital after April, 1942, and who were treated according to the therapeutic regimen described. The control group consisted of those patients who were admitted prior to April 1, 1942, and who were not treated according to any particular therapeutic regimen. For the most part these patients received a high carbohydrate diet. An occasional omentopexy was done.



Of the entire series of 647 patients, 112 were omitted from consideration in this study because of doubts concerning the validity of the diagnosis. There remained 224 patients in the experimental series and 311 patients in the control group. Autopsy or biopsy material was available on approximately 65 per cent of these patients. The remainder of the diagnoses were based upon unmistakable clinical and laboratory data. (See Table II.)

TABLE II CASE MATERIALS

	EXPERIMENTAL (4/1/42 TO 1/1/46)	CONTROL (4/1/35 TO 4/1/42)	BOTH (4/1/35 TO 4/1/46)
Total number of hospital admissions	5621	6181	12029
Diagnosis of cirrhosis	263	384	647
Diagnosis doubtful	39	73	112
Total number of patients with cirrhosis	224	311	535
Incidence (per cent of total admissions)	0.40	0.49	0.45

Although the number of patients in each of the two series was thought to be sufficiently large to insure their comparability, an effort was made to compare the two series in as many respects as possible.

The incidence of the disease among the total hospital admissions, the sex, incidence of the disease, the age of the patients, their occupations, the presenting complaints, the initial physical findings, and the laboratory data were approximately the same in the two series of patients. Because of the rationale upon which the therapeutic regimen is based, the dietary and alcoholic histories as well as the size of the livers deserve special consideration.

*Alcohol Consumption*—Objective data regarding alcohol consumption before admission are difficult to secure. Both the patient and the physician are likely to color the information as the result of their own past experiences. The following arbitrary classification of alcohol consumption is the basis for the present evaluation.

One plus, occasional consumption of alcoholic beverages but not to the point of intoxication, two plus, average daily consumption of one or two highballs or two to four glasses of beer, three plus, average daily consumption of a pint of hard liquor or four quarts of beer, four plus, all others.

TABLE III HISTORY OF ALCOHOLISM IN PATIENTS WITH CIRRHOSIS

	EXPERIMENTAL		CONTROL		BOTH	
	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
No data	33	15.5	94	60.1	127	34.4
Data	180	84.5	62	39.9	242	65.6
Total	213	100.0	156	100.0	369	100.0
Alcoholism						
Denied	12	6.7	1	1.6	13	5.4
1+	24	13.4	0	0.0	24	9.9
2+	20	11.1	3	4.8	23	9.5
3+	21	11.7	21	33.9	42	17.3
4+	103	57.7	37	59.7	140	57.9
Total	180	100.0	61	100.0	241	100.0

Most would probably agree that those characterized by three and four plus alcohol consumption should be classified as chronic alcoholics. The data presented in Table III reveal that 70 to 75 per cent of the patients in these two series were chronic alcoholics.

*Dietary Histories*—Accurate data on dietary habits are equally difficult to secure and must also necessarily involve subjective factors both on the part of the patient and the interrogator. Nonetheless an attempt was made to evaluate the diets in approximately half the patients. In Table IV it will be observed that 75 per cent or more of the patients were existing on inadequate food intake. Deficiencies in protein, fresh fruits, and vegetables as well as total calorie intake usually were obvious. Such deficiencies were correlated with alcohol consumption. Patients readily admitted that when they were drinking they did not eat.

TABLE IV DIETARY HISTORY OF PATIENTS WITH CIRRHOSIS

	EXPERIMENTAL		CONTROL		BOTH	
	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
No data	109	51.2	143	91.7	252	68.3
Data	104	48.8	13	8.3	117	31.7
Total	213	100.0	156	100.0	369	100.0
Adequate	16	15.4	0	0.0	16	13.7
Average or fair	8	7.7	0	0.0	8	6.8
Inadequate	80	76.9	13	100.0	93	79.5
Total	104	100.0	13	100.0	117	100.0

The 20 or 25 per cent of the patients whose consumption of alcohol was not considered to be excessive and whose diet was not necessarily inadequate are of considerable interest. While it is undoubtedly true that some of these individuals may have been inaccurate in relating their histories, it is equally certain that lesions of the liver indistinguishable clinically or anatomically from those of typical alcoholic cirrhosis occurred in the absence of gross dietary defects or excessive alcohol consumption. Vague histories of previous jaundice were obtained in some of these patients. Whether or not these were instances of infectious hepatitis cannot be stated with any certainty.

TABLE V LIVER SIZE

	EXPERIMENTAL (%)	CONTROL (%)	BOTH (%)
Size unknown	16.6	42.9	26.6
Size recorded	83.4	57.1	73.4
Total	100.0	100.0	100.0
No enlargement	23.2	20.1	22.1
1 cm. below costal margin (Rt. M.C.L. *)	3.8	6.8	4.8
2 cm. below costal margin (Rt. M.C.L.)	17.0	16.9	17.0
3 cm. below costal margin (Rt. M.C.L.)	23.7	24.7	24.0
4 cm. below costal margin (Rt. M.C.L.)	24.2	16.9	21.8
5 cm. below costal margin (Rt. M.C.L.)	5.4	11.2	7.4
6 cm. or more below costal margin	2.7	3.4	2.9
Total	100.0	100.0	100.0

\*M.C.L. Mid-costal line

*Size of Liver*—Enlargement of the liver was one of the most constant physical findings. More than 75 per cent of the patients had definitely palpable livers. In most instances the enlargement was considerable, as is evident upon inspection of Table V. It is presumed that such enlargement was the result of fat infiltration and hypertrophy of liver cells. Lipotropic substances should be of value under these circumstances.

## FINDINGS

*Survival*—The fate of the patients in the two groups as of Oct. 1, 1947 is summarized in Table VI. One hundred sixty nine or 75.5 per cent of the experimental group were known to be dead as compared with 266 or 85.5 per cent of the control group. This comparison permits no conclusion regarding the merits of the therapeutic regimen since the period of observation for the experimental group was several years shorter than that of the control group. The observed periods of survival after the onset of initial symptoms varied from 210 to 730 months in the experimental group as compared with 78.0 to 176.0 months in the control group. The average period of survival for the period of observation was 40.2 and 122.0 months respectively.

TABLE VI FATE OF PATIENTS WITH CIRRHOSIS (10/1/47)

	EXPERIMENTAL		CONTROL	
	NUMBER	PER CENT	NUMBER	PER CENT
Dead	169	75.5	266†	85.5
Living	37	16.5	6	2.0
Fate unknown	18	8.0	39	12.5
Total	224	100.0	311	100.0

61.3 per cent autopsied

16.9 per cent autopsied

*Mortality*—It was not possible to utilize all these cases since the cause of death in some instances was not thought to be directly or indirectly related to the liver disease. In still other instances the lack of adequate data regarding the time of onset of the disease precluded use in this comparison. The experimental series was thus reduced to 139 patients thought to have died of liver insufficiency after a definite period of clinical disease as compared with a similar series of 197 cases in the control group. (See Table VII.)

The percentage of patients in each of the two groups dead at any given time after the onset of the initial symptoms is compared graphically in Fig. 1. No difference between the two groups of patients is apparent. The maximum

TABLE VII SELECTION OF CASES FOR STUDY

	EXPERIMENTAL	CONTROL
Complicating diseases	9	7
Iradequate data	21	6
Available for comparison	139	197
Total	169	206

known period of survival in the experimental group to date is slightly less than six years as compared with more than sixteen years in the control group

A definite time (within a month) of onset of ascites was elicited in 100 instances in the experimental group and in 154 instances in the control group

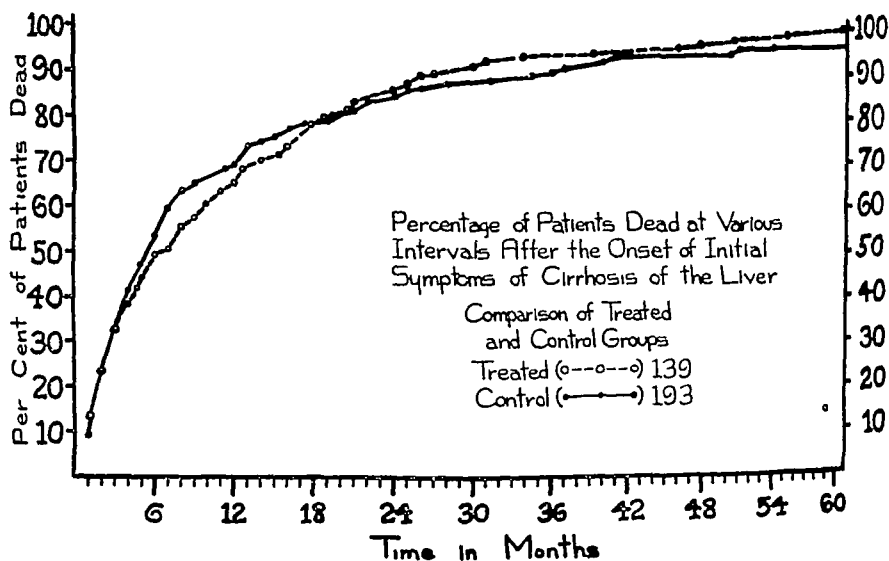


Fig 1

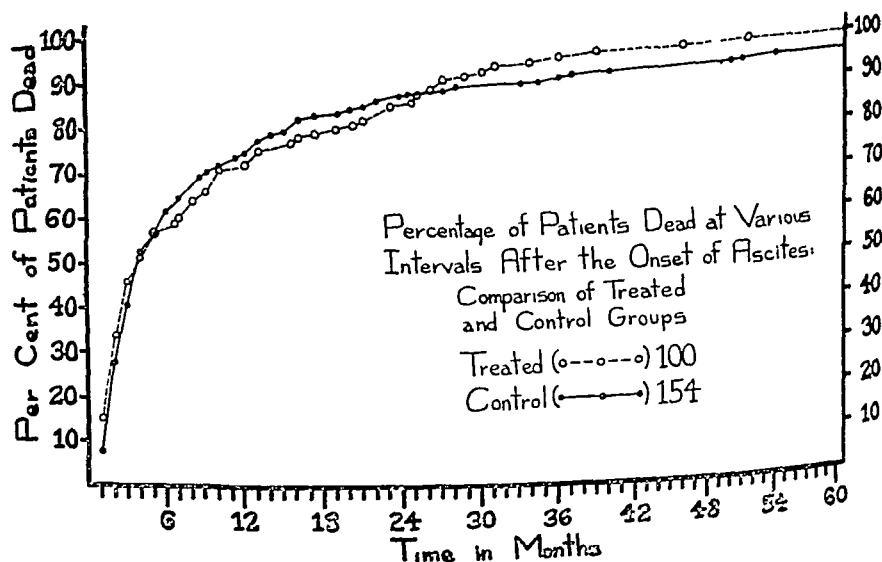


Fig 2

The percentage of patients dead at any given time after the onset of ascites is compared for the two groups in Fig 2. The results are in no wise different from those described in the preceding paragraph.

A clear cut history of the onset of jaundice thought to be associated with the patient's cirrhosis, was elicited in forty six experimental subjects and in sixty three control patients. It is of interest that several patients gave a history of jaundice ten or more years prior to the obvious onset of cirrhosis.

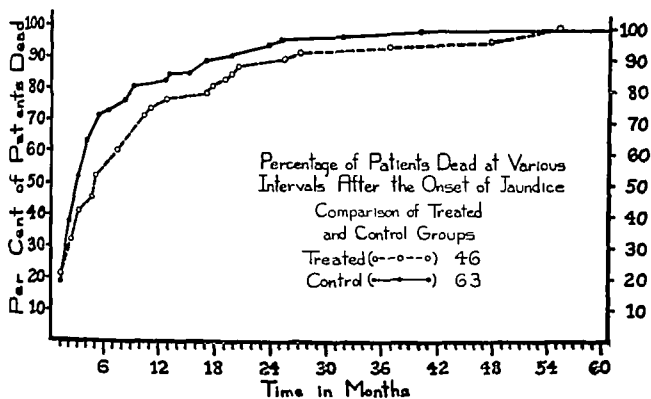


Fig. 3

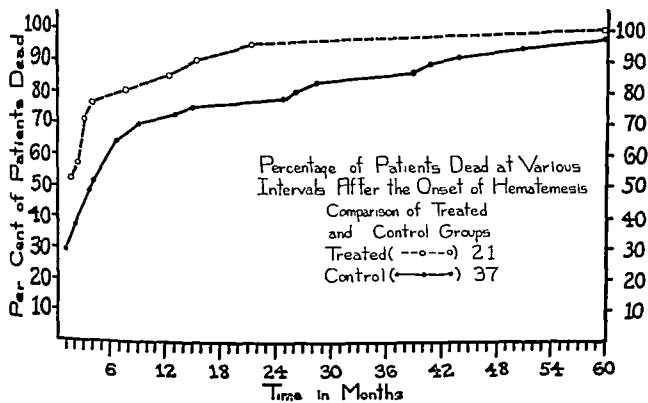


Fig. 4

Such attacks of jaundice were not interpreted as heralding the onset of the patient's cirrhosis. The percentage of patients of the two groups of patients known to be dead at various times after the onset of jaundice is compared in Fig. 3. Again there is no basis for assuming any difference in the life expectancy of individuals in the two groups.

Finally, the percentage of patients dead at various intervals after the onset of gastrointestinal bleeding is compared in Fig. 4. The number of patients available for comparison was markedly less than in the previously described groups—twenty-one in the experimental group and thirty-seven in the control group. It will be noted that the treated group fared less well than the control group. Since more than half of the treated patients were dead in less than two months it is hardly reasonable to assume that therapy could have had any influence on these patients.

#### COMMENT

It is clear that in spite of preferential treatment with regard to supervision and follow-up care, the experimental group fared no better than the control group so far as survival is concerned. Several possible explanations for the failure of the therapeutic regimen to prolong life deserve consideration.

- (1) The regimen was not adequately followed by the patients.
- (2) The disease was so far advanced as not to be amenable to therapy.
- (3) The therapeutic regimen was inadequate in one or more respects.
- (4) Initial liver damage, possibly by factors other than dietary defects, had rendered the organ incapable of utilizing the materials supplied.

(1) Since 60 to 75 per cent of the patients in this series were chronic alcoholics it is probable that difficulty was encountered in enforcing the therapeutic regimen when the patients were not under observation in the hospital. In addition it is important to point out that many of these patients were without adequate resources for the purchase of food and other essentials. To avoid this difficulty, as previously indicated, prolonged hospitalization was utilized freely. Attempts at the evaluation of the adequacy of therapy in individual cases were made but was found to be impracticable.

(2) Evaluation of the stage of the patient's disease was also difficult. The size of the liver is ordinarily considered to decrease as the cirrhosis advances. This we found was not strictly true. A large percentage (70 to 75 per cent) of the patients studied, however, did have definite hepatic enlargement suggesting hypertrophy and/or fat infiltration. Such patients may reasonably be expected to respond more quickly and adequately than those with small, hard, intensely fibrosed livers.

(3) Analysis of the therapeutic regimen in the light of clinical and laboratory observations of the past few years suggests some desirable changes. The importance of supplying adequate amounts of an adequate variety of amino acids should be emphasized. The patient with cirrhosis is invariably suffering from depletion of tissue protein as well as blood protein. The keen competition for individual amino acids that may ensue under such circumstances by a multiplicity of body processes is well known. Fat transport and utilization as well as repair of damaged liver parenchyma are at the mercy of the amino acid supply.

The amino acid supply may be influenced profoundly by the presence of pancreatic disease, edema or atrophy of the intestinal tract, and the palatability

of the diet (fat content) These obstacles may be at least partially overcome by supplementing the oral consumption of protein with parenteral amino acids, at least at the onset of therapy

Some doubt may be cast upon the wisdom of strict limitation of the fat intake The relative importance of deficiencies of the essential fatty acids, excessive cholesterol intake, and adverse effect upon the protein intake cannot be decided readily The great importance of protein would certainly argue in favor of considerably more freedom in the fat intake than has been permitted in this therapeutic experiment so as to assure optimal protein intake

Only a limited number of studies are available upon which one might base any accurate statements regarding choline requirements or tolerance in man It is known that the requirement is influenced by other dietary factors that is protein (methionine cystine), growth, and so forth Assuming the requirement in man to be comparable to that in the dog or chick it is reasonable to assume that the human requirement is not 15 to 30 Gm daily<sup>1</sup> The average diet of man contains 15 to 40 Gm daily<sup>1</sup> If the toxic effect of choline on man is comparable to that experienced by chicks and mice man would be expected to experience minimal toxic effects from 15 to 20 Gm of choline daily<sup>1</sup> Assuming the daily diet of the patients studied to have contained 40 Gm of choline, even with the maximum dose used (8 Gm) no toxic manifestations would have been anticipated It is possible that a marked increase in choline dosage is indicated since Kaplan and Chaikoff state that as much as 3 Gm of choline daily are required to cure fatty livers in dogs if such fat infiltration is permitted to develop<sup>3</sup> Upon the basis of body weight this is comparable to doses of 15 to 30 Gm daily in a human being weighing 60 kilograms Much of the animal experimentation has been preventive rather than curative and is of little value in determining the dose needed in the treatment of human beings with established liver lesions

The methionine requirement is probably influenced by other dietary factors growth and so forth as is the choline requirement Very little specific information is available to guide one in the matter of dosage

While the use of supplements of the B complex (brewers yeast crude liver extracts, and so on) was avoided in the treatment of the series of patients described, there can be no doubt that such supplements are indicated in view of the multiple evidences of malnutrition observed in patients with cirrhosis Aside from the correction of multiple specific deficiencies, the salutary effect upon the patient's appetite is of extreme value It was reasoned that mobilization of liver fat would be attended by improvement in appetite, and relief of specific deficiencies by the ingestion of an adequate diet This may be true but valuable time may be lost at a critical stage in the patient's illness

(4) Primary damage to the liver may so impair its ability to discharge its responsibilities for fat metabolism that a fatty liver will ensue no matter how adequate the supply of dietary essentials may be Such primary damage might be produced by many agents or methods Experimentally phosphorus or carbon tetrachloride may produce such impairment of function even though accompanied by or preceded by the use of lipotropic factors If the influence of the

noxious agent is eliminated and enthusiastic therapy begun, the damage may be reversible. Unrecognized or inadequately treated damage of this sort in the human subject may be very difficult or impossible to treat successfully when it ultimately comes to the attention of the clinician.

#### CONCLUSIONS

Low fat diets supplemented by 1 to 8 Gm of choline and one quart of skimmed milk daily (0.9 Gm methionine) failed to influence the life span of patients with cirrhosis of the liver, even though these patients were much more closely supervised than the control series.

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## DISTRIBUTION OF GOLD IN THE ANIMAL BODY IN RELATION TO ARTHRITIS

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ALTHOUGH gold and gold salts have had wide clinical application in diseases of the skin in tuberculosis and of late in rheumatoid arthritis their mode of action is undetermined. Worth while information about the pharmacology of gold salts has been gained particularly through the application of a micro method developed by Block and Buchanan<sup>1</sup> in the metabolic studies of Freyberg and associates as well as in the investigations by Denko and Anderson<sup>2</sup> Cortell and Richards<sup>3</sup> and Hartung and Cotter<sup>4</sup>. The following contains experimental data on the distribution of gold given as radioactive material and first used by Ely.<sup>11</sup>

### METHOD

Au<sup>198</sup> (half life 2.7 days) produced in a pile by the (n,  $\gamma$ ) reaction was obtained from the Atomic Energy Commission with a specific activity of about 2.5 mc per milligram. Radioactive gold sodium thiosulfate was synthesized from the radioactive gold and administered intravenously. The tissue samples and excreta were weighed and ligated in nitric acid and hydrogen peroxide, the gold was then quantitatively removed by a process of electro deposition<sup>12</sup> developed by Dunn of this laboratory. The punchlets with the radioactive gold were counted by means of a bell jar Geiger Muller counter with a thin mica window. All data presented were corrected for half life.

### RESULTS

The excretion of Au<sup>198</sup> was studied in two white rats. The fractions of initially administered drug excreted during the first eight days are given in Table I. After the first week excretion became very slow. Further excretion studies are now in progress.

*Gold Distribution in Rats*—Distribution experiments were set up using rats in which quantitative recovery of the administered gold was attempted. In these experiments all excreta were saved the gold being recovered from this and from the whole carcass (minus samples removed). When such a procedure was followed, 97.75 per cent of the total gold administered could be recovered. Duplicate samples of each tissue were taken as a further check on the accuracy of the method employed.

The discrepancies indicated for bone and synovials cannot be explained completely. In the case of synovials values tend to become higher at longer

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TABLE I

TIME (DAY)	GOLD THIOSULFATE EXCRETION (%)	
	URINE	FECES
1	8.4	3.5
2	2.7	1.0
3.4	2.8	0.8
5.6	1.1	0.6
7.8	1.0	0.8
Total in 8 days	16.3	6.7
Administered 1 mg 0.1 millicurie		

TABLE II DISTRIBUTION OF GOLD SODIUM THIOSULFATE IN THE RAT, QUANTITATIVE RECOVERY

TISSUE	PER CENT OF INJECTED DOSE PER GRAM TISSUE	
Muscle	101	0.78
Skin	246	2.36
Liver	663	6.60
Kidney	5.780	5.820
Spleen	1.310	1.180
Heart	152	1.87
Intestine	220	2.10
Lung	131	1.29
Synovialis	295	4.83
Articular cortex (bone)	212	2.57
Bone	0.0057	0.014
Blood	3.87	3.47
Carcass		21.31
Blood		52.20
Excreta		24.24
Total recovered		97.75%

Duplicate samples used Intestinal sample taken from ileum Administered dose 12 mc  
 1 mg gold) Rat sacrificed forty-eight hours after administration  
 Articular cortex refers to cartilage of joint surface and compact bone directly under it

intervals, and there is also the factor of small sampling. Synovial samples from rats average 5 to 10 mg, and small amounts of evaporation could easily account for significant weight loss in such samples. Any such error is greatly increased when data are expressed in terms of grams.

When the accuracy of the technique was ascertained by such quantitative recoveries a series of fifteen rats was employed, each rat receiving 2 ml of radiogold sodium thiosulfate solution containing 10 mg of gold and approximately 1 mc of radioactivity. The amount of radioactivity varied with the time of injection and was in all cases exactly known. The animals were sacrificed at intervals of two to eight days. Before sacrifice these animals were perfused with physiologic saline solution through the proximal end of the inferior vena cava and were bled through the distal end of the same vessel, in some cases however, the saline was introduced directly into the heart while bleeding took place through the inferior vena cava.

The agreement between respective determinations was fair in most cases however, some organs showed considerable fluctuations in gold content—notably the kidney and spleen. The time interval had little to do with the values of

any given tissue, which would seem to indicate that gold is held rather firmly by the respective tissues once it has left the blood stream. As can be seen in Table I, the amount excreted is small after the fourth day.

Gold distribution data were taken on ten rabbits at different time intervals. Before sacrifice the rabbits were perfused with normal saline through the proximal end of the inferior vena cava and bled through the distal end of the same vessel. There was a general agreement between respective determinations although some organs showed considerable fluctuations in gold content. A typical set of the results obtained for the concentration of  $\text{Au}^{198}$  in various types of tissue is given in Table IV.

TABLE III DISTRIBUTION OF GOLD IN RATS

Tissue	PER CENT OF INJECTED DOSE PER GRAM TISSUE
Kidney	8.72
Spleen	1.14
Thyroid	.74
Synovials	.67
Liver	.67
Tendon	.52
Skin	.41
Articular cortex (bone)	.15
Testicle	.28
Heart	.21
Muscle	.07

These data represent the averages on fifteen rats.

TABLE IV GOLD DISTRIBUTION IN THE RABBIT DOSE 404 MICROCURIE  $\text{Au}^{198}$  (10 MG  $\text{Au}$ )  
SACRIFICED SEVENTEEN DAYS AFTER ADMINISTRATION

TISSUE	PER CENT OF INJECTED DOSE PER GRAM TISSUE
Kidney	.57
Spleen	.14
Liver	.01
Adrenal	.02
Tendon	.023
Skin	.016
Small intestine	.015
Synovials	.014
Bone	.014
Blood	.014
Marrow	.013
Lymph node	.012
Aorta	.012
Testicle	.01
Heart	.009
Articular cortex (bone)	.007
Lung	.006
Muscle	.002

In the course of this work specific activities of several tissues were ascertained for which the uptake of gold salts had not been demonstrated previously, such as the iris, aqueous and vitreous humors and brain. All these organs contained gold quantities in the order of  $10^{-4}$  per cent of the injected amount per gram of tissue.

A comparison of the data given for rabbits and rats shows great variance which is due to the greatly increased dilution of the administered dose in the rabbit. The average weight of the rats used was 150 grams, while that of the rabbits equaled 2.5 kilograms. When correction was made for this factor the results were comparable, as can be seen in Table V.

TABLE V COMPARISON OF GOLD DISTRIBUTION IN RABBITS AND RATS

TISSUE	RAT	RABBIT
Kidney	8.72	6.14
Spleen	1.14	2.3
Liver	.67	1.16
Synovialis	.67	.23
Tendon	.52	.38
Skin	.47	.28
Articular cortex	.45	.12
Heart	.21	.15
Muscle	.07	.03

Values expressed in per cent total dose injected per gram tissue

The similar relative order is interrupted by the synovial sample in the rat, which may be falsely high.

*Gold Uptake in Chemical Synovitis*—Chemical arthritis was produced in five rabbits by the intra-articular injection of 0.5 cc of a solution, composed of U. S. P. turpentine 3 parts and diethylether 1 part, into the knee joint of one extremity. This was repeated in five days. Ten days after the initial injection the animals were given the radiogold thiosulfate by the intravenous route, and five days later the rabbits were sacrificed for assay after first being perfused with 0.89 per cent saline. The rabbits had not been walking since twenty-four hours following the initial treatment. The joints were swollen to twice the normal size and were tender and hot to the touch.

On autopsy the synovialis showed proliferation to a moderate degree and the articular surfaces were dull and roughened.

As can be seen from Table VI, in all cases the chemically inflamed tissues took up a significantly larger amount of gold salt than the normal tissue. From previous experience with the distribution of gold in the blood components it was known that the white blood cells contained considerable amounts of thiosulfate, and it was thought that this fact might account for the higher level in

TABLE VI CHEMICAL ARTHRITIS

N Tendon	0.04	0.06	0.28	0.23	0.26
P Tendon	0.13	0.16	1.24	.27	.04
N Synovial	0.15	0.18	0.74	.16	.09
P Synovial	0.58	0.84	2.25	—	.05
N Cortex	0.09	0.18	.05	.13	.11
P Cortex	.02	.05	.035	.19	
N Muscle	0.029	.07			
P Muscle	.03	.113			
PUS	0.28	0.33			.05

Rabbits were given 1.5 mc ten days after turpentine injection. Values are expressed in per cent total dose per gram tissue.

P and N refer to pathologic and normal respectively.

the inflamed tissues, however pure pus from sterile abscesses was found to have less activity per gram than the tissues under consideration and in tissues such as bone and tendon large exudative responses were not noted. It was clear from this that even though the inflammatory elements might be responsible for part of the concentration they could not possibly account for all and that in the presence of inflammation the tissues themselves soaked up more of this salt.

In view of these facts we wondered about the specificity of this phenomena for joint structures. It was found that apparently the reaction would occur outside of joints, for when the same chemical mutant was injected into muscle and a sterile abscess formed the muscle wall of the abscess would also accumulate the salt. This perhaps makes the accumulation in the joints less noteworthy but nonetheless real.

*Human Experimentation*—It was possible\* to carry out a preliminary distribution experiment on a human arthritic subject. The patient selected was a 57 year old white woman with rheumatoid arthritis of a moderately advanced stage and of approximately ten years duration. Pain upon motion was present in both knees; movement was only slightly restricted. The patient had never received chrysotherapy. She was given 1010 microcuries of radiogold as the sodium thiosulfate salt representing 25 mg of gold. Twenty-four hours after administration, biopsy of the left knee was done; specimens of skin, superficial fascia and fat, deep fascia and fat, synovial fluid, synovial membrane and muscle were taken. Of the tissues taken the synovialis was by far the most active with the synovial fluid next in activity. This indicates that in human beings with rheumatoid arthritis the synovialis is at least somewhat of a concentrator of gold.

TABLE VII GOLD DISTRIBUTION IN RHEUMATOID ARTHRITIS

Synovialis	014
Synovial fluid	0094
Muscle	0013
Superficial fascia	000366
Deep fascia	000596
Skin	00074

It is interesting to note that the synovialis is eighteen times higher in gold content than skin. If we compare the gold concentration ability of human and rat skin the value (.014 per cent) for synovialis is in the range one would expect for kidney—18 times .47 (rat skin) equals 8.46 (value for rat kidney).

## CONCLUSIONS

$Au^{198}$  is a suitable radioactive isotope for studying the action of gold on the animal body. Excretion and distribution data obtained with this isotope

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conform with previous observations using stable gold. Synovials, tendon, and articular cortex in chemical arthritis show a greater uptake of gold than similar tissues of normal joints, and this was shown to be nonspecific since the muscle wall of sterile abscesses exhibits the same phenomenon. Passage of gold from the blood stream into the central nervous system and ocular structures of rabbits has been demonstrated.

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# THE EFFECT OF NUTRITION ON THE TUMOR RESPONSE IN ROUS CHICKEN SARCOMA

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## INTRODUCTION

NUMEROUS observations have confirmed the fact that nutrition may influence the genesis and growth of tumors in rats and mice. The search for requirements in the nutrition of malignant tissue should reveal findings that are valid regardless of the nature of the tumor studied or the kind of animal used as well as more limited findings. Such information might well include the facts that adequate caloric intake has been shown necessary for tumor growth<sup>1</sup> and that vitamins such as pantothenic acid and riboflavin have been found stimulative<sup>2</sup> whereas choline may be inhibitory.<sup>3</sup>

## EFFECT OF NUTRITION IN ROUS SARCOMA

Little and co-workers<sup>4</sup> showed that folic acid has a marked effect on the tumor response in Rous chicken sarcoma. The effectiveness of folic acid free diets in preventing the tumor response of baby chicks and the rapidity and regularity with which the response occurred in adequately nourished chicks led us to our present study of the effect of each of the different constituents in a synthetic diet for chickens.

The data to be presented confirm observations made on rats and mice to the effect that malignant tissue may utilize vitamins such as pantothenic acid and riboflavin for growth. Our results also indicate that nicotinamide and choline acid may be stimulative. However, folic acid appears to be the only nutrient required to the extent that tumor response is prevented by its absence.

*Method for Demonstrating the Effect of Nutrition*—In our tests the extent to which each nutrient influenced tumor response was determined by comparing the incidence of tumors in groups of chicks fed the complete diet with the incidence of tumors in groups fed the same diet without the nutrient. By comparing observations made on the eighth, tenth, twelfth, fourteenth, and sixteenth day the effect of nutrition was evaluated for all stages from the usual time of first appearance of tumors to the time when 90 to 100 per cent of control birds showed tumors. The response with and without nutrient was calculated from the combined results of different tests where more than one test was carried out. The percentage of birds showing tumors when fed diets with the nutrient was divided by the percentage showing tumors when fed the same diet without the nutrient. The nutrients which showed no stimulative effect gave values of approximately 1.0.

*Relation of Size of Inoculum to Tumor Response*—An even distribution of tumor response over the eight days selected for observation of results was readily obtained by controlling the size of the inoculum. As shown in Table I, there is a direct correlation between the size of inoculum and the length of the latent period.

We used homogeneous amples of frozen virus prepared by blending fresh tumor tissue in a Waring mixer and weighing 2 Gm. amounts into sterile Petri plates to be stored in a dry

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TABLE I TUMOR RESPONSE OBTAINED WITH HOMOGENEOUS PREPARATION OF INOCULUM KEPT FROZEN FOR VARIOUS PERIODS

AMOUNT OF INOCULUM USED (0.25 ML DOSE)	INOCULUM KEPT FROZEN (DAYS)	TOTAL NUMBER OF CHICKS USED	NUMBER OF CHICKS THAT GREW TUMORS	PERCENTAGE OF TAKES	AVERAGE LATENT PERIOD (DAYS)
10 mg	Fresh	21	21	100.0	7.90
	16	16	15	93.7	7.93
1 mg	Fresh	25	25	100.0	9.50
	16	16	15	93.7	9.00
	24	190	173	90.1	9.03
0.1 mg	Fresh	25	23	92.0	12.04
	16	17	16	94.1	11.20
	24	190	157	82.6	11.08
0.01 mg	Fresh	23	13	56.5	12.84
	16	20	8	40.0	14.00

ice chest until used. Preliminary suspensions containing 10 mg in a 0.25 ml dose were prepared by adding 50 ml of 2 per cent peptone solution to 2 Gm of tumor tissue. A Ten Brock grinder was employed, and further dilutions were made in peptone solution.

In our experiments the desired length of latent period was obtained with 1 mg of inoculum. The frozen virus was found to be stable for at least a month. It is of interest that Reinhard and co-workers<sup>5</sup> found a similar relationship between the size of inoculum and the length of the latent period for growth of a transplantable mouse adenocarcinoma.

One day old New Hampshire Red chicks which were the progeny of a selected flock (as described in our previous report<sup>4</sup>) were injected in the right breast with 0.25 ml of the suspension containing the 1 mg dose. The feathers were removed from the breast area before inoculation. Groups of ten birds were placed on diets with and without each nutrient tested. Nutrients which were of interest because of possible stimulative effect were retested several times. The chicks were maintained in electric brooders at 90° F, water and food were supplied ad libitum. Approximately 400 chicks (ten to twenty per group) were used in each experiment.

The basal diet contained 53 per cent Cerelose,\* 22 per cent alcohol extracted casein, 4.3 per cent salt mixture, 3 per cent calcium gluconate, 8 per cent gelatin, 4 per cent Ruffex,† 3 per cent soybean oil, 0.25 per cent cholic acid, 0.45 per cent cystine, 200 mg per cent choline chloride, 3 mg per cent calcium pantothenate, 3 mg per cent nicotinamide, 0.5 mg per cent pyridoxine, 0.3 mg per cent thiamin chloride, 0.03 mg per cent biotin, 0.5 mg per cent riboflavin, 100 mg per cent inositol, 5 mg per cent para-aminobenzoic acid, 0.2 mg per cent folic acid, 5 mg per cent vitamin E, 0.2 mg per cent vitamin K, 3,500 units per cent vitamin A, and 200 units per cent vitamin D. The diet with nutrient was usually the complete diet. A commercial chick starter gave the same tumor response as the complete synthetic diet. In studies of substitutes for soybean oil, the diet with nutrient contained the substitute and the basal diet was soybean oil free. In these tests the vitamins A, D, E, and K were added in propylene glycol.

The chicks were observed daily beginning with the eighth day and continuing through the sixteenth day at which time adequately nourished groups showed 90 to 100 per cent tumors. Wing tags applied on the sixth or seventh day were used to identify individual birds. The increasing sizes of tumor were recorded (as previously described) for evidence as to the accuracy of first observations. Tumors recorded as questionable were counted in determining the response whenever this observation was confirmed by subsequent findings.

\*Glucose monohydrate Fisher Scientific Co. Pittsburgh, Pa.

†Purified cellulose containing 70 per cent α-cellulose and 30 per cent other cellulose. Fisher Scientific Co. Pittsburgh, Pa.



## EXPERIMENTAL RESULTS

Three distinct types of results have been obtained with nutrients which demonstrated a definite stimulative action on tumor growth. While with and without values of approximately 10 were obtained in tests on nutrients which did not stimulate tumor growth (A) constant values of approximately 30 were obtained in tests of riboflavin, (B) descending values of 130 62 46, 26 21 were obtained in tests of nicotinamide and (C) ascending values of 130 400 630 890, 930 were obtained in tests of folic acid. While other nutrients gave results similar to those illustrated in A and B, folic acid was the only nutrient giving the result C.

*The Effect of Vitamins From Liver*—Table II shows the effect of nine water soluble vitamins on the tumor response of chicks to Rous sarcoma virus. Folic acid produced the greatest effect and para aminobenzoic acid and biotin produced the least effect. Nicotinamide and calcium pantothenate influenced the rate of growth more than the final incidence. riboflavin did not change the rate of growth but did influence the incidence at all stages.

TABLE II EFFECT OF WATER SOLUBLE VITAMINS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

VITAMIN	AMOUNT IN DIET	NUMBER OF CHICKS (A/B†)	NUMBER OF TESTS	RESPONSE WITH VITAMIN				
				RESPONSE WITHOUT VITAMIN				
				8	10	12	14	16
Thiamin	3 mg/kg	10/10	1	30 0	15	13	11	11
Riboflavin	5 mg/kg	39/37	4	21	30	31	31	31
Pyridoxine	5 mg/kg	30/30	3	20	29	18	14	13
Nicotinamide	30 mg/kg	29/29	3	130	62	46	26	21
Calcium pantothenate*	30 mg/kg	29/18	3	240	110	26	20	18
Inositol	1 Gm/kg	29/25	3	130	12	11	10	10
Para aminobenzoic acid	50 mg/kg	19/19	2	14	11	10	08	08
Biotin	0.3 mg/kg	29/27	3	11	18	12	11	11
Folic acid	2 mg/kg	30/32	3	130	400	630	890	930

\* Vitamin deficient chicks were revived with complete diet beginning on the tenth day.  
† a/b Total chicks used to determine effect of diets (a) with and (b) without the vitamin specified.

Since deficiencies of thiamin, pyridoxine and calcium pantothenate caused serious loss of weight, chicks fed diets without these vitamins were revived with complete diet beginning on the tenth day. In previous work with folic acid it had been shown that the stimulative effect of restoring this vitamin on the tenth day does not become apparent for at least seven days. This practice was adopted in the case of thiamin, pyridoxine and calcium pantothenate deficiencies to permit survival of the birds for the duration of the test. The responses with and without one of these vitamins for the twelfth, fourteenth and sixteenth day may or may not be influenced by restoring the vitamin on the tenth day.

Since liver is a rich source of still unidentified vitamins, several fractions of liver were tested in the presence of the nine purified vitamins for possible stimulative effect on the tumor response of chicks to Rous sarcoma virus. None of the liver fractions influenced the rate of growth or incidence of tumor to an extent which would indicate the presence of additional factors for tumor growth.

*The Effect of Vitamins A, D, E, and K*—The results of tests of diets with and without vitamins A, D, E, and K are shown in Table III. None of these vitamins stimulated tumor growth when present in the diet. The values 0.8, 0.6, and 0.5 suggest that the oil-soluble vitamins may slightly retard tumor growth. It is of interest that diets with and without the combination of soybean oil and vitamins A, D, E, and K influenced the tumor response to a greater extent than did the diets with and/or without the oil-soluble vitamins alone.

TABLE III. EFFECT OF OIL SOLUBLE VITAMINS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

VITAMIN	AMOUNT IN DIET	NUMBER OF CHICKS (A/B*)	NUMBER OF TESTS	RESPONSE WITH VITAMIN				
				RESPONSE WITHOUT VITAMIN				
				8	10	12	14	16
Combined A	35,000 I U/kg	19/19	2	0.8	1.0	1.0	1.0	1.0
D	2,000 I U/kg							
Combined A	35,000 I U/kg	10/10	1	0.5	1.3	1.1	1.1	1.1
D	2,000 I U/kg							
E	50 mg/kg	10/8	1	0.6	1.2	1.4	1.2	1.2
K	2 mg/kg							
E	50 mg/kg	10/10	1	0.5	1.0	1.5	1.1	1.0
K	2 mg/kg							
Combined Soybean oil	5 per cent	20/23	2	1.0	2.6	2.3	2.0	1.6
A, D, E, K	As above							

\*A/B Total chicks used to determine effect of diets (A) with and (B) without the vitamin specified.

*The Effect of Various Fats and Oils*—Table IV shows the results of tests in which twelve different substances were tested in a soybean oil free diet. None of these substances stimulated tumor growth. Soybean lecithin, cod liver oil, and linoleic acid appeared to retard tumor growth when present in the diet. Most of the other substances in the group showed this effect to some degree.

TABLE IV. EFFECT OF FATS AND OILS IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

NUTRIENT*	AMOUNT IN DIET (%)	NUMBER OF CHICKS (A/B)	NUMBER OF TESTS	RESPONSE WITH NUTRIENT				
				RESPONSE WITHOUT NUTRIENT				
				8	10	12	14	16
Beef liver fat	5	10/10	1	1.0	1.0	0.7	1.0	1.0
Cholesterol	1	54/54	3	1.0	1.2	0.7	0.7	0.8
Coconut oil	5	30/30	3	0.1	1.3	1.1	1.3	1.3
Cod liver oil	3	10/10	1	1.0	0.7	0.4	0.3	0.6
Corn oil	5	10/10	1	0.5	0.5	0.8	1.0	1.0
Crisco	5	10/10	1	1.0	1.2	0.5	0.8	0.8
Lanolin	3	10/10	1	1.0	0.7	0.5	0.6	0.8
Lard	5	10/10	1	0.0	0.7	0.5	0.8	0.8
Linoleic acid	3	30/30	3	0.0	0.5	0.7	1.2	1.3
Sodium oleate	3	30/30	3	0.1	1.2	1.0	1.4	1.4
Soybean lecithin	1	20/20	2	0.0	0.8	1.3	0.6	0.6
Soybean lecithin	3	10/10	1	0.0	0.5	0.5	1.0	1.0
Soybean oil	5	20/20	2	2.0	1.3	1.0		

\*The basal diet was oil-free. Vitamins A, D, E, and K were added in propylene glycol.  
 ‡a/b Total chicks used to determine effect of diets (a) with and (b) without the substance specified.

*The Effect of Cholic Acid*—Table V shows the results of tests of diets with and without cholic acid, sodium chloride gelatin, calcium gluconate, and Ruffex. Diets with and without cholic acid influenced tumor growth in much the same way as did diets with and without riboflavin. Three times as many tumors developed when cholic acid was present in the diet.

TABLE V EFFECT OF CHOLIC ACID IN DIET ON ROUS SARCOMA (RATIO OF PER CENT INCIDENCE ON THE COMPLETE DIET TO PER CENT INCIDENCE ON THE DEFICIENT DIET)

NUTRIENT	AMOUNT IN DIET (%)	NUMBER OF CHICKS (A/B)	NUMBER OF TESTS	RESPONSE WITH NUTRIENT				
				RESPONSE WITHOUT NUTRIENT				
				8	10	12	14	16
Cholic acid	0.5	20/20	2	30.0	32	38	31	31
Sodium chloride	1	15/15	1	0.4	0.6	0.9	0.8	0.9
Gelatin	8	10/10	1	3.0	3	1.4	1.2	1.2
Combined								
Gelatin	8							
Calcium gluconate	3	10/10	1	3.0	0	1.6	1.6	1.4
Ruffex	4	65/65	4	1.6	1.0	0.9	0.9	0.8

a/b Total chicks used to determine effect of diet: (a) with and (b) without the substance specified

#### SUMMARY

The effect of nutrition on the tumor response in Rous chicken sarcoma was determined by comparing observations of chicks fed synthetic diets with and without each nutrient. Tumor response was stimulated by the presence of folic acid, nicotinamide, calcium pantothenate, riboflavin, and cholic acid in the diet. Folic acid was the only nutrient required to the extent that tumor response was prevented by its absence from the diet.

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# THE USE OF ANTAGONISTS OF PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

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SINCE Woods<sup>1</sup> described the interference of *p*-aminobenzoic acid on the action of sulfonamides, the antagonism between compounds promoting growth and those which inhibit growth has received increasing attention. Analogues of pteroylglutamic acid (folic acid) have been studied extensively. The antagonistic activity of these compounds in the growth of *Streptococcus faecalis* R has been examined by Hutchings and Stokstad.\* Experiments leading to the demonstration of a stimulatory effect of pteroylglutamic acid and an inhibitory effect of its antagonists on Rous chicken sarcoma have been reported by Little and co-workers<sup>4</sup> and by Woll.<sup>5</sup>

In the present report we wish to present more recent findings with regard to the effect of choice of antagonists and method of administration on the outcome of experiments with Rous chicken sarcoma. Table I contains a list of the antagonists of pteroylglutamic acid which we have tested for ability to control Rous chicken sarcoma. Both the abbreviated names of compounds (such as used in our first report on Rous sarcoma) and the complete chemical names are shown as an aid in referring to reports dealing with the synthesis of these compounds. The antagonists which we have found most useful, for reasons to be discussed, are the 4-amino-pteroylaspartic acid and the 4-amino-pteroyl (D-glutamic acid which were synthesized by Mowat and others,<sup>10\*</sup> and the 4-amino-pteroylglutamic acid synthesized by Seeger, Smith, and Hultquist.<sup>†</sup>

## PROCEDURE

In producing Rous chicken sarcoma we have employed homogeneous samples of frozen virus prepared by blending fresh tumor tissue in a Waring mixer and weighing 2 Gm. amounts into sterile Petri plates to be stored in a dry ice chest until used. Preliminary suspensions containing 10 mg. in a 0.25 ml. dose were prepared by adding 50 ml. of 2 per cent peptone solution to 2 Gm. of tumor tissue. A Ten Broeck grinder was employed, and further dilutions were made in peptone solution. The size of inoculum used in our experiments was determined by titration of the virus for even distribution of tumor responses over a period of time from the eighth to the sixteenth day of the test. The frozen virus was found to be stable for at least a month with respect to the effect of concentration of virus on the time distribution of tumor responses. Table II illustrates the method employed for standardizing the virus.

## HANDLING OF CHICKS

New Hampshire Red chicks which were the progeny of a selected flock (as described in our previous report)<sup>4</sup> were injected in the right breast with 0.25 ml. of the suspension containing the dose required for the desired distribution of tumor responses. The feathers were removed from the breast area before inoculation. Groups of ten birds were compared with

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\*Lederle Laboratories Division.

†Calco Chemical Division.

TABLE I. LIST OF ANTAGONISTS OF PTEROYLGLUTAMIC ACID TESTED ON ROUS CHICKEN SARCOMA

ABBREVIATED NAME	CHEMICAL NAME	RFF EVIDENCE
Pteroylaspartic acids	N [4 {[(2 amino 4 hydroxy 6 pteridyl) methyl] amino} benzoyl] aspartic acid	6
4 Amino folic acid or 4 Amino pteroylglutamic acid	N [4 {[(2 4 diamino 6 pteridyl) methyl] amino} benzoyl] glutamic acid	3 7
V methyl folic acid* or V methyl pteroylglutamic acid	V [4 {V [(2 amino 4 hydroxy 6 pteridyl) methyl] Δ methylamino} benzoyl] glutamic acid	3 8
Δ methyl pterois acid*	Δ [V [(2 amino 4 hydroxy 6 pteridyl) methyl] V methylamino} benzoyl] glutamic acid	3 8
4 Amino V methyl folic acid* or 4 Amino V methyl pteroylglu tamic acid	N [4 {V [(2 4 diamino 6 pteridyl) methyl] V methylamino} benzoyl] glutamic acid	3 9
4 Amino N methyl pterois acid	Δ [V [(2 4 diamino 6 pteridyl) methyl] V methylamino} benzoyl] glutamic acid	9
4 Amino pteroylaspartic acid*	Δ [4 {[(2 4 diamino 6 pteridyl) methyl] amino} benzoyl] aspartic acid	10
4 Amino folic acid with d( ) glu tamic acid* or 4 Amino pteroyl d( ) glutamic acid	V [4 {[(2 4 diamino 6 pteridyl) methyl] amino} benzoyl] d( ) glutamic acid	10

Designation used in our first report on the effect of folic acid and its antagonists on Rous chicken sarcoma

TABLE II. TITRATION OF ROUS SARCOMA VIRUS FOR EVEN DISTRIBUTION OF TUMOR RESPONSES

AMOUNT OF TISSUE IN INOCULUM (0.05 ML.)	TOTAL CHICKS USED	TOTAL TESTS MADE	PERCENTAGE OF CHICKS WITH TUMORS				
			DAY				
			8	10	1	14	16
10 mg.	37	4	64	43	90	90	97
1 mg.*	140	14	14	30	38	71	91
100 μg	140	14	0	6	26	45	71
10 μg	140	14	0	1	3	14	31

This amount of virus gave the desired distribution of tumor responses

as many untreated controls. The chicks were maintained in electric brooders at 90° F. water and food were supplied ad libitum. Baby chicks varying from 1 to 9 days old were tested for ability to resist the toxic effect of different antagonists of folic acid.

A daily record of observations was made from the eighth day of the test to the twentieth day. The growth of tumors from the time of first appearance was recorded in the manner previously described. This information enabled us to demonstrate retardation of tumor growth when inadequate doses of antagonists were used. In the table presented in this paper, chicks showing the slightest evidence of tumor have been counted in with those showing the usual tumor response characteristic of untreated groups.

#### EXPERIMENTAL RESULTS

Table III shows the results of experiments with 4 amino pteroylaspartic acid. This antagonist of folic acid was administered (1) by daily intraperitoneal injection to chicks which were 2 days old at the start, (2) by daily intraperitoneal injection to chicks which were 8 days old at the start and (3) by feeding to chicks which were only 1 day old at the start. It is apparent from Table III that under the conditions in (1) the toxicity of the chemical for the chicks interfered with its value as an inhibitor of tumor growth. Under the conditions in (2) the chicks were more resistant to the toxic effect of doses inhibitory to tumor growth. Under the conditions in (3), chicks only 1 day old at the start could

TABLE III USE OF 4 AMINO PTEROYL ASPARTIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITH OUT TUMOR (%)	UNTREATED CHICKS WITH OUT TUMOR (%)
<i>(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks</i>					
2	0.02	10	0	44	0
2	0.1	10	40	50	10
2	0.2	20	75	80	5
2	0.2	10	80	50	0
2	0.2	10	60	50	0
<i>(2) Antagonist Administered by Daily Intraperitoneal Injection to Week Old Chicks</i>					
8	0.2	10	0	60	10
8	0.4	10	10	44	10
<i>(3) Antagonist Administered in Diet of Baby Chicks</i>					
1	20/kg	15	0	0	9
1	80/kg	20	10	75	5

TABLE IV USE OF 4 AMINO PTEROYL D( ) GLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
<i>(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks</i>					
3	0.01	10	0	22	0
2	0.02	10	20	42	0
3	0.1	10	30	75	0
2	0.2	20	95	66	5
2	0.2	10	80	50	0
<i>(2) Antagonist Administered by Daily Intraperitoneal Injection to Week Old Chicks</i>					
8	0.1	10	20	37	10
8	0.2	10	30	42	10
8	0.4	10	60	100	10
<i>(3) Antagonist Administered in Diet of Baby Chicks</i>					
1	20/kg	15	0	0	9
1	80/kg	20	0	55	5

TABLE V USE OF 4 AMINO PTEROYL GLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START	DOSE OF CHEMICAL (MG)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
<i>(1) Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks</i>					
2 days	0.01	10	100	—	0
3 days	0.1	10	100	—	0
<i>(2) Antagonist Administered in Diet of Baby Chicks</i>					
1 day	20/kg	20	100	—	9
<i>(3) Antagonist Administered by Daily Intraperitoneal Injection to Adult Birds</i>					
6 weeks	1.0	10	10	62	0
7 weeks	1.0	10	0	60	0
8 weeks	1.0	10	0	40	0

be treated successfully with 80 mg of 4 amino pteroylaspartic acid per kilogram of diet without toxic effect. The diet was the regular commercial chicken ration.

Table IV shows that similar results were obtained with 4 amino pteroyl d( ) glutamic acid. Daily injections of the doses of chemical required to inhibit tumor growth resulted in impairment of health and eventual loss of birds when the chicks were 2 to 3 days old at the start. Chicks 8 days old at the start showed greater resistance to the toxic effects of doses inhibitory to tumor growth. Chicks only 1 day old at the start showed no toxic effect when fed 80 mg of 4 amino-pteroyl d( ) glutamic acid per kilogram of diet.

Table V shows that 4 amino pteroylglutamic acid (4 amino folic acid)<sup>4</sup> is suitable for use in the treatment of adult birds only. In a series of experiments we succeeded in neutralizing the toxicity of 4 amino pteroylglutamic acid for baby chicks by giving simultaneous injections of pteroylglutamic, pteroyl diglutamic or pteroyltriglutamic acid. It was found that 0.25 mg doses of pteroylglutamic acid protected 60 per cent of chicks, 0.25 mg doses of pteroyl triglutamic acid protected 50 per cent of chicks and 0.25 mg doses of pteroyl diglutamic acid protected 10 per cent of chicks against the toxic effect of 0.01 mg doses of 4 amino pteroylglutamic acid. This antagonist did not prevent tumor growth in baby chicks thus protected from its toxic effect.

TABLE VI USE OF 4 AMINO N METHYL PTEROYLGLUTAMIC ACID IN CONTROLLING ROUS CHICKEN SARCOMA

AGE OF CHICKS AT START (DAY)	DOSE OF CHEMICAL (MG)	TOTAL CHICKS USED PER GROUP	DIED OF TOXICITY OF CHEMICAL (%)	TREATED CHICKS WITHOUT TUMOR (%)	UNTREATED CHICKS WITHOUT TUMOR (%)
<i>Antagonist Administered by Daily Intraperitoneal Injection to Baby Chicks</i>					
2	0.02	10	0	22	0
2	0.1	10	80	50	10
2	0.2	10	70	33	0
-	0.2	20	55	77	5

Table VI shows the results of experiments with 4 amino N methyl pteroyl glutamic acid. When administered to baby chicks by daily intraperitoneal injection, this antagonist demonstrated toxicity and also inhibited tumor growth. It was not administered in the diet because of lack of material. The toxicity of 4 amino-N methyl pteroylglutamic acid appears to be similar to that of 4-amino pteroylaspartic acid and 4 amino pteroyl d( ) glutamic acid. These antagonists when injected produce symptoms resembling those caused by maintaining chicks on folic acid free diets. We have found that chicks showing severe symptoms as a result of injection with these antagonists quickly revive when treatment is discontinued.

#### DISCUSSION

It is evident from our experiments that Rous chicken sarcoma may be controlled by regulating the amount of pteroylglutamic acid in the tissues of the chicken either by use of synthetic folic acid free diets<sup>4</sup> or by use of suitable

chemical antagonists. Severe deficiencies of the vitamin, whether caused by lack of folic acid in the diet or by the injection of powerful antagonists such as 4-amino-pteroylglutamic acid, regularly result in death of birds. It is well known that the amount of folic acid required to maintain the normal health of chickens is relatively small. According to Oleson<sup>11</sup> 0.5 mg per kilogram of diet is suboptimum, 1 to 2 mg are optimum, and 2 to 5 mg are superoptimum.

Our experiments indicate that at least two of the chemical antagonists of folic acid (4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid) and possibly a third (4-amino-N-methyl-pteroylglutamic acid) may be appropriate tools for controlling the amount of folic acid available in the body for tumor growth without depriving the body of the amounts of vitamin required for normal health. When the treated animal is a rapidly growing chick, these chemicals appear to be more serviceable than 4-amino-pteroylglutamic acid. When the treated animal is an adult chicken, 4-amino-pteroylglutamic acid is not only serviceable, but may well be the chemical of choice inasmuch as it is effective in small doses.

The toxicity of these chemicals appears to be due solely to the severity of the vitamin deficiency which may result from too intensive treatment. Our experiments indicate that treatment by mouth is the method of choice, since tumor growth may be inhibited by this method in a greater percentage of animals without toxic effect. Evidently the tumors of Rous Chicken sarcoma are more directly dependent for their growth on the folic acid furnished by the diet than are the normal tissues of the chick. Since chicks do not develop severe deficiencies of the vitamin in the time required to demonstrate inhibition of tumor growth by use of folic acid free diets (as shown in our previous report),<sup>4</sup> it is possible that the health of such chicks is protected by stores of the vitamin which are not available to tumors of this type. If such is the case, the most desirable method of treatment would be the administration in the diet of chemicals which would antagonize the vitamin present in food. We have shown that 4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid can produce this result.

#### SUMMARY

Two chemical antagonists of pteroylglutamic acid (4-amino-pteroylaspartic acid and 4-amino-pteroyl-d(-)glutamic acid) have been found capable of inhibiting tumor growth in baby chicks inoculated with Rous sarcoma virus when the chemicals were fed ad libitum at a concentration of 80 mg per kilogram of diet. These chemicals and also 4-amino-N-methyl-pteroylglutamic acid were active in inhibiting tumor growth in baby chicks when injected. Toxic effects resembling the effect of severe vitamin deficiency occurred when the chemicals were injected, but not when the chemicals were fed.

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# TREATMENT OF THE TYPHOID CARRIER STATE

## TRIAL OF TWO CHEMOTHERAPEUTIC PROCEDURES

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ALTHOUGH the medical literature contains many reports of unsuccessful attempts to clear up the chronic typhoid carrier state by the use of chemotherapeutic agents, it nevertheless is deemed worth while to record the results of the two studies presented here since they represent failures to confirm previously published favorable reports.

Although typhoid fever in New York State exclusive of New York City is rapidly reaching the vanishing point (fifty-two cases reported in 1947), the residual chronic carriers living in the state continue to offer the threat of further outbreaks of the disease. A total of 465 known chronic carriers are listed on the roster of the New York State Department of Health, and it is estimated that some 2,500 carriers actually survive in the state at the present time.<sup>1</sup> A chemotherapeutic agent effective in clearing up the typhoid carrier state would thus be a real boon to public health as well as to the individual carriers involved.

*Trial of Tin Compound in the Treatment of Typhoid Carriers*—The report by Reitler and Marberg<sup>2</sup> presenting apparently clear-cut evidence of therapeutic benefits afforded by a tin compound (heptadekylaldehyde stannoxysterate) in the treatment of typhoid fever and, more specifically, in the cure of two typhoid carriers seemed to warrant further clinical trial of the compound. Supplemental information<sup>3</sup> indicated that three of four definitely proved chronic typhoid carriers were cleared of their carrier condition by administration of this drug. Accordingly in December, 1944, twenty-one chronic typhoid carriers, inmates of two New York State mental hospitals,\* were selected for treatment. Preliminary stool examinations† of the carriers established the persistence and constancy of their carrier status. They were then treated with successive courses of this tin preparation‡ according to the schedule suggested by Reitler.<sup>2</sup>§ Pertinent data concerning the carriers utilized in this and the subsequent study are presented in Table I. In Fig. 1 are illustrated the time schedule of therapy and the results of stool examination.

It will be noted that none of the established carriers were cleared even temporarily of typhoid bacilli. Carrier 3, who appears to be an exception, evidently recovered spontaneously before treatment was started. Carriers 5, 9, and 16 demonstrated intermittency in their carrier condition, but this characteristic

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\*Willard and Harlem Valley State Hospitals.

†All bacteriologic examinations in these studies were performed at the Division of Laboratories and Research, New York State Department of Health.

‡The drug used was kindly furnished by the firm Chemica Ltd., Haifa, Palestine, which markets the drug under the trade name Aldestan.

§Each course consisted of the daily administration by mouth of ten tablets (0.02 Gm. of active ingredient per tablet) for ten days followed by a rest period of one week.

TABLE I TYPHOID CARRIERS INCLUDED IN STUDY DATA RELATING TO DURATION OF THE CONDITION

CAR- RIER	AGE (YR)	SEX	HISTORY OF TYPHOID FEVER YEARS PRIOR TO STUDY	BACTERIO- LOGICALLY PROVED DURATION OF CARRIER STATE (YR)	LENGTH OF RESI- DENCE IN MENTAL HOSPITAL (YR)	VI AGGLU- TINA- TION TITLE AT TIME OF STUDY	BACTERIO- CULTURE TITLE	GALL BLADDER X RAYS
1	69	F	Unknown	6	40	1 10	-	Poor concentration
2	65	F	Unknown	5	26	1 10	-	-
3	55	M	5	5	14	1 40	-	-
4	59	F	Unknown	5	24	1 10	F	Poor concentration
5	47	F	Unknown	8	29	1 40	Could not determine	-
6	48	F	Unknown	7	20	1 40	F	Stones
7	67	M	Unknown	5	5	1 0	C	-
8	61	F	Unknown	8	27	1 10	Could not determine	Calcified cyst Poor concentration
9	71	F	Unknown	4	47	1 10	-	-
10	44	F	Unknown	6	25	1 40	-	-
11*	66	F	Unknown	3	19	1 40	E	-
12	49	F	8	8	13	1 10	Imp V	-
13	50	F	Unknown	3	13	1 10	V	-
14	30	F	8	8	16	-	Imp V	-
15	58	F	30	9	11	1 10	B	-
16	37	F	Unknown	1/2†	10	Neg	-	-
17	66	F	1	1	10	1 20	Imp V	-
18	44	F	Unknown	1/2†	17	Neg	W Form	-
19	83	F	Unknown	7†	12	Neg	A	-
20	55	F	Unknown	1†	11	Neg	Imp V	-
21	61	F	Unknown	3	13	1 20	Imp V	-

\*These carriers also were treated with penicillin and sulfathiazole in a subsequent study described below.

†These individuals were shown to be still carrying *Bacillus typhosus* two and one half years after the study.

existed before treatment was started. Routine blood and urine examinations were carried out periodically on these carriers throughout the course of treatment in order to determine whether the drug was producing any untoward reactions but no significant difficulties were encountered.

The value of this procedure in curing typhoid carriers was given a severe test since, for the most part, it could be shown that these individuals were carriers of long duration, even though the mental status of the patients precluded the getting of a history of typhoid fever in all but four instances. Cholecystectomy had not been performed on any of the group. X-ray studies of four of the carriers indicated that either gall stones were present or that the gall bladder functioned poorly. Nevertheless, on the basis on these findings one can hardly attribute any benefit to this therapeutic procedure.

*Trial of Penicillin Plus Sulfathiazole in the Treatment of Typhoid Carriers*—The report by Bigger<sup>4</sup> demonstrating the existence of the synergistic effect of sulfathiazole and penicillin on *Escherichia typhosa* in vitro, led to the clinical trial of these drugs by Comerford and co-workers in the treatment of two typhoid carriers. This carefully planned study seemed to demonstrate beyond doubt the cure of these carriers, both through the prompt disappearance of typhoid bacilli from the stools during the course of treatment and the continued

absence of these organisms in daily stool cultures for six months thereafter when the study was terminated. In addition, a striking drop in the typhoid Vi antibody titer of the blood occurred over this period of time. The only reservation one might have concerning the results of this work is that one of the two carriers studied was convalescent from typhoid fever and had carried the organism for only one year, and the other was a chronic carrier who persisted in excreting typhoid bacilli in the stool for ten months following cholecystectomy. Thus neither case was typical of the usual chronic typhoid carrier of long standing with a markedly sclerosed gall bladder containing stones, and it seemed possible that the course of treatment outlined would be ineffective in eradicating the infection in such individuals. Nevertheless, this therapeutic technique appeared to warrant further trial. Since that time an additional paper<sup>6</sup> has reported apparent success in curing a single typhoid carrier with massive doses of penicillin alone.

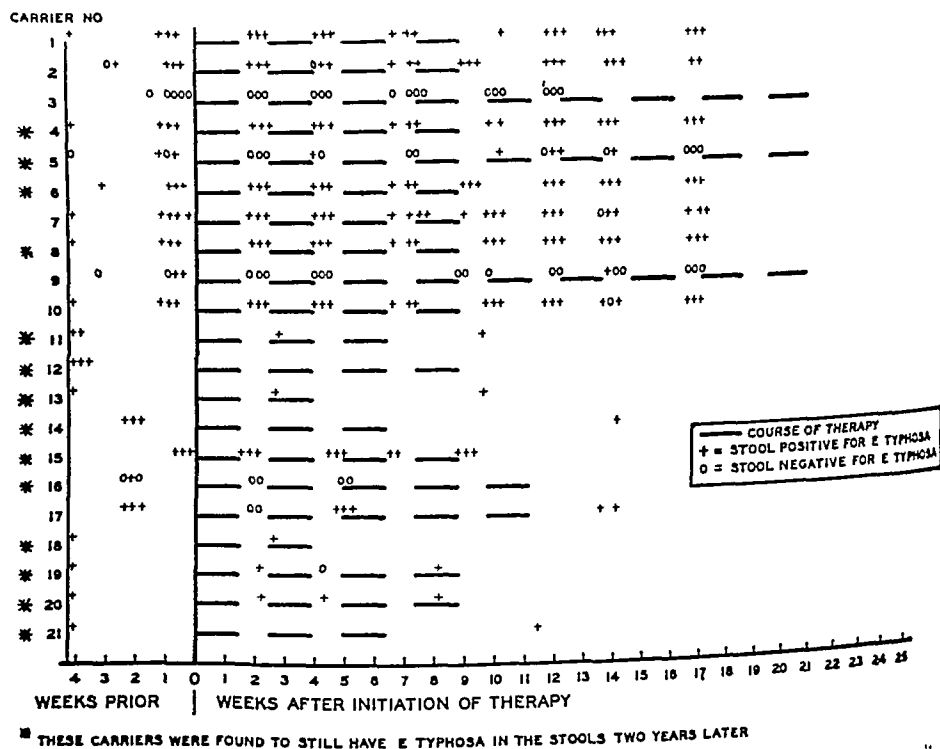


Fig 1—Treatment of typhoid carriers with tin compound schedule of therapy and results of stool examinations

The schedule of therapy utilized by Comerford and co workers was dictated by in vitro studies which demonstrated the optimal concentrations of sulfathiazole and penicillin needed for inhibition and destruction of the typhoid bacillus. With slight modification, the same schedule was utilized in the present study.

A total of eight chronic carriers, inmates of New York State mental hospitals,\* were utilized in this study which was started in August, 1947. (This

\*Willard and Harlem Valley State Hospitals

series represents Carriers 6, 8, 11, 12, 13, 14, 15, and 21 of the first study) Accumulated records indicated the long time chronic nature of their carrier state Daily stool specimens for a period of ten days prior to initiation of treatment confirmed the persistence of the carrier state and the constancy of positive stool findings None of these individuals were urinary carriers For an eight day period sulfathiazole was given by mouth 1 Gm every four hours, and crystalline sodium penicillin, in doses of 1 000 000 units was administered intramuscularly every six hours Studies of the penicillin blood level\* showed uniformly high levels of penicillin one half hour after administration of the drug which reached a peak of 61 units per milliliter of blood during the fifth day of treatment Samples collected immediately before successive doses were administered showed at all times more than 0.49 unit of penicillin per milliliter The results of bacteriologic examination of the stools from these carriers before, during, and subsequent to treatment are illustrated in Fig 2

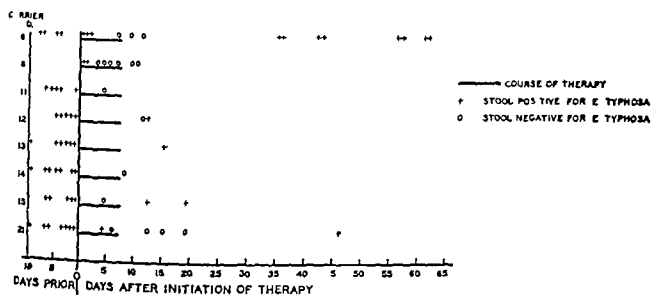


Fig 2—Treatment of typhoid carriers with penicillin and sulfathiazole record of stool examinations and schedule of therapy

Other pertinent data concerning these carriers already have been presented in Table I. It is evident that in each instance typhoid bacilli disappeared completely from the stool for a period. In two instances careful study failed to reveal their presence for several weeks after completion of the course of treatment. However, the organisms reappeared subsequently and hence in no instance was a carrier actually cured. Several of these individuals had been shown previously to have gall stones thus accounting perhaps for some of the difficulties in eradicating the infection.

Although none of the carriers treated in this study were permanently cured it is possible that this therapeutic procedure might have some application in the treatment of the typhoid carrier state which persists occasionally following cholecystectomy or in eradicating the carrier condition early in its development in convalescent cases of typhoid fever. However, it is doubtful that the procedure outlined would be effective in curing the infection in the

\*These tests were performed in the laboratory of Dr George A. Taplin University of Rochester Rochester N. Y.

average chronic carrier, although more prolonged therapy with larger doses of the two drugs might be more fruitful. These findings with respect to the effect of penicillin plus sulfathiazole on *E typhosa* are in keeping with those reported by Hardy<sup>7</sup> who observed a similar bacteriostatic action on *E typhosa* with sulfadiazine in nineteen chronic typhoid carriers. It would seem that the only therapeutic measure of proved value for the eradication of the typhoid carrier state is that of cholecystectomy. Experience in New York State<sup>8</sup> indicates that in 68 per cent of chronic carriers undergoing cholecystectomy cure was obtained.

#### SUMMARY AND CONCLUSIONS

Treatment of twenty-one chronic typhoid carriers with the tin compound heptadekylaldehyde stannoxystearate (Aldestan) failed to demonstrate any effect on the presence of *E typhosa* in the stool.

The administration of penicillin and sulfathiazole to eight chronic typhoid carriers caused the disappearance of typhoid bacilli from the stool in each individual. In two instances the organisms were absent for at least two weeks following discontinuance of treatment. However, they reappeared in the stool of all the carriers.

The authors would like to acknowledge the cooperation of Dr. Kenneth Keill, Superintendent, Willard State Hospital, and Dr. Alfred M. Stanley, Superintendent, Harlem Valley State Hospital, in making these studies possible.

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## THE OCCURRENCE OF SALMONELLA BLEGDAM IN LOUISIANA\*

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*SALMONELLA blegdam* was first isolated in 1929 at State Serum Institute<sup>1</sup> Copenhagen, Denmark, from the blood of a patient in the Blegdam Hospital<sup>2</sup> suffering from pneumonia of the right lower lobe. This organism was not described, however, until 1935 by Kauffmann.<sup>3</sup> In 1941, Tourner<sup>1</sup> again found *S. blegdam* in the blood of a patient in Shanghai, China. Atkinson and associates<sup>4</sup> recorded in 1944 the occurrence of this bacterium in blood and feces of four cases of enteric like fever in soldiers in New Guinea. During the first year of the United States reoccupation of the Philippines Stevens isolated four strains of *S. blegdam* and noted his findings in 1946. Two of these four isolations were from the blood of American infantry soldiers with symptoms of paratyphoid infections, one was from the feces of a soldier without apparent enteric fever and the other one was from an ulcerative lesion on the ankle of a Filipino patient with no gastrointestinal symptoms. Cobley and Wilson<sup>5</sup> in 1946 reported a case of *S. blegdam* septicemia and suppurative pericarditis with recovery in an Australian soldier. This *Salmonella* was isolated both from the blood and pericardial fluid and identified by Atkinson and co-workers. Fenner and Jackson<sup>6</sup> described in 1946 fifty cases of enteric fever due to *S. blegdam* again in Australian soldiers from New Guinea. The diagnosis was established in seventeen cases by the identification of the organism by Atkinson and associates and in the remainder of the cases by clinical epidemiologic, and serologic findings. In 1947, Atkinson and associates recorded additional *S. blegdam* isolations from blood and feces of soldiers and natives in New Guinea and Bougainville Island but as well as can be ascertained seventeen of these strains were obtained from the cases reported by Fenner and Jackson. Five strains from mice and one strain from a guinea pig in a laboratory stock suffering from an epidemic also were found by Atkinson and co-workers.

We wish to report at this time the isolation in December 1947 of a strain of *S. blegdam* from the feces of a patient in the Southern Baptist Hospital presenting an enteric type of fever and in extensive erythema. During January 1948 an isolation of *S. blegdam* also was made from the blood of a patient in the Touro Infirmary with upper respiratory and gastrointestinal symptoms thought at first to be a viral infection. It is of clinical interest that repeated blood counts during the period of hospitalization showed a persistent leucopenia with depression of the granulocytic series.

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We are grateful to Dr. Erich Seligman and Dr. Ivan Saphir, National Salmonella Center, New York, N. Y., for the final identification of both strains of *S. blegdam* isolated by us. Acknowledgment is also made to Miss Louise Cargile, Southern Baptist Hospital, and to Miss Madeleine Page, Touro Infirmary, for their technical assistance.

TABLE I DATA PERTINENT TO INTERPRETATION OF BACTERIOLOGIC RESULTS

Type of illness	SOUTHERN BAPTIST HOSPITAL				TOKYO INFIRMARY			
	Enteric type of fever, extensive erythema				Upper respiratory and gastrointestinal symptoms, fever			
Source of isolation	Feces, 11 days after hospitalization				Blood, 7 days after hospitalization			
Other specimens examined	Two blood cultures (second week), results negative for Salmonella				Stool cultures (second and third weeks) and urine culture (second week), results negative for Salmonella			
Agglutination tests	FEBRILE HOSPITAL DAY				FEBRILE HOSPITAL DAY			
	3	9	12	20	5	11	9	69
Typhoid	Ty O	0	0	0	Ty O	Pos 1 40	Pos 1 160	Pos 1 320
	Ty H	0	0	0	Ty H	Pos 1 160	Pos 1 320	Pos 1 320
	Para A	0	0	0	Para A	Pos 1 80	0	Pos 1 160
	Para B	0	0	0	Para B	Pos 1 40	0	Pos 1 160
	Bi	0	0	0	Bi	0	0	0
	Pr X19	0	0	0	Pr X19	0	0	0
Typhoid	NB Serum collected 7 weeks after hospitalization showed widal pos 1 800 according to results obtained at National Salmonella Center				TUBE LIVING ANTIGENS			
					S blegdam	X	Pos 1 1280	Pos 1 420
					S typhosa	X	X	Pos 1 640
					S typhi murium	X	X	Pos 1 160
					S oranienburg	X	X	0
					S virginia	X	X	Pos 1 160
Typhoid					S javiana	X	X	Pos 1 320
					S newington	X	X	Pos 1 160
					Proteus X19	X	X	Pos 1 160
								0
Typhoid paratyphoid vaccination	No record of previous typhoid vaccination, negative agglutination tests indicate no vaccination				1943, 3 injections, April, 1945, 3 injections, April, 1946, booster, Feb., 1947, booster			
	Discharged 41 days after admission to hospital				Discharged 10 days after admission to hospital			
Epidemiology	Patient's husband was a member of Armed Services stationed in various parts of South Pacific Theatre during World War II, two stool cultures on his hand were negative for <i>S. blegdam</i> , patient had eaten sea food prior to onset of symptoms				Patient (student nurse) visited home 1 to 2 weeks prior to onset where brother and mother had been ill, cases diagnosed "flu", diarrhea, nausea, vomiting, but no fever, duration 3 to 4 days, brother served in U S Army 1944-45 in New Guinea New Hebrides Islands, New Caledonia, etc., no laboratory studies available on either mother or brother			



We feel that these findings are worthy of reporting since we have been unable to find any reference in the literature to the previous isolations of *S blegdam* in the United States. In correspondence on December 24, 1947 with Sehlgman,\* we were informed that this species of *Salmonella* had never before been found in this country. Of further interest is that the second case from which this rare species was isolated again in New Orleans simulated clinically the original one described by Kauffmann<sup>3</sup> in Copenhagen. These two strains of *S blegdam* are thought to be the first isolated from anyone in the United States and from individuals who had never left this country.

**Biochemical Activities**—The isolation of *S blegdam* from both blood and feces followed the usual procedure in the study of enteric pathogens. In Kligler's agar, the production of acid and gas in butt, alkaline reaction on slant and hydrogen sulfide occurred. It failed to ferment lactose, sucrose, salicin, dulcitol and inositol. Fermentation with gas production occurred in glucose, mannitol, maltose, xylose, sorbitol, arabinose and rhamnose. Indole was not formed. Urea was not split. Milk showed no coagulation. Gelatin was not liquefied.

**Antigenic Structure**—According to Beigey's Manual *S blegdam* possesses the formula IX, XII g,m,q —. Because this organism possesses the somatic antigen IX, it is classified in the serological Group D. It is a monophasic flagellated bacillus existing only in phase 1. In our laboratories we were able to identify the somatic antigens only. The identification of the flagellar antigens was made by the National Salmonella Center. (See Table I.)

#### SUMMARY

*S blegdam* was isolated for the first time in Louisiana and probably in the United States by Newton from the feces of a patient suffering with enteric type of fever and extensive erythema during the fifth week after onset.

The second isolation of *S blegdam* in Louisiana was by Holt from the blood of a patient with upper respiratory and gastrointestinal symptoms closely resembling clinically those of the patient from which *S blegdam* was first isolated by Kauffmann as well as the type of *Salmonella* fever described by Fenner and Jackson in which complications developed. The organism was found during the second week of the infection in a student nurse.

Neither patient had traveled outside the United States. Both patients gave histories of contact with members of the Armed Forces who had previously served in New Guinea and various posts in the South Pacific Theatre during World War II. No epidemiologic facts, however, could be established on the carrier state of these two contacts or on other possible sources of infection.

Serologically, results obtained on routine febrile and *in vitro* agglutination tests by Holt failed to be of value in the diagnosis of this infection and led to confusion. Attention is called to the close antigenic relationship of *S blegdam* to *S typhosa*.

The final identification of this organism was by analysis of the antigenic structure of which it is composed.

Routine vaccination against typhoid-paratyphoid infections as used in this country afforded no protection against *S. blegdam* in one of the patients.

From a review of the available literature we could find recorded the isolation of only thirty-one strains of *S. blegdam* from human sources prior to this; additional two strains now being reported by us.

Diagnosis of this *Salmonella* infection is best established by the isolation of *S. blegdam* from blood, stool, urine, or any source involved.

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# EXPERIMENTAL THERAPY OF GENERALIZED TORULOSIS IN RATS WITH STREPTOMYCIN

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INDIANAPOLIS IND

IT HAS been pointed out previously in an excellent monograph by Cox and Tolhurst<sup>1</sup> that if any treatment favorably influences the course of torulosis it is as yet unknown. However, a localized lesion may heal spontaneously.

Many forms of therapy have been used to little or no avail. The acriflavine dyes<sup>2,3</sup> have been administered by several routes including the intrathecal route, with eventually fatal results. Beck and Voyles,<sup>4,5</sup> Hamilton and Tyler, Marshall and Teed,<sup>6</sup> and many others have used the sulfonamides and potassium iodide with few and very uncertain beneficial results. Jones and Klinck in therapeutic experiments in mice demonstrated that sulfadiazine and penicillin were of no value. The subjects of clinical cases reported by Haiford and co-workers<sup>7</sup> and Cox and Tolhurst<sup>1</sup> did not benefit from penicillin. Stoddard and Cutler<sup>8</sup> and Hoff<sup>9</sup> have amply demonstrated the ineffectiveness of the injection of the killer organisms and the other *Torula* antigens. Mezey and Fowler<sup>11</sup> report one case with a short follow up in which some benefit was derived from the use of intravenous 5 per cent alcohol and dextrose solution. Shapiro and Neal<sup>12</sup> used colloidal silver and immune rabbit serum intraspinally without favorable result. X ray therapy has been tried without benefit.<sup>13</sup>

Since little or no success with any of the afore mentioned therapeutic agents has been achieved a therapeutic test using streptomycin has been carried out in rats.

In vitro titrations of streptomycin against *Torula*\* showed little anticytoprotozoic activity. Apparently there was no inhibition of the same strain used in this experiment. However Whiffen and co-workers<sup>14</sup> discovered a highly effective antibiotic against *Torula* in the liquors in which streptomycin was prepared. This agent was effective in vitro but was extremely toxic in vivo and therefore could not be used in animal experiments.

Forty seven medium sized albino rats were selected for this trial since it had been shown previously that the rat is easily infected with *Torula* through intraperitoneal inoculation. In addition it does not succumb to the infection too readily. An inoculum was prepared with the same strain in the same fashion described by Beck and Voyles<sup>5</sup> in 1946. Each rat received 100 000 organisms intraperitoneally.

One week after the inoculation with the standardized *Torula* suspension treatment was begun. The rats were separated into two groups the control group numbering twenty one and the treated group numbering twenty six. Both groups were maintained under identical conditions and were fed the same type food. In no way was a differentiation made except in that one group was treated with 3,000 units of streptomycin in three doses every twenty four hours for twenty one days. The drug was given subcutaneously over the abdomen.

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No attempt was made to check the levels in the blood. At the end of the three week period, two of the control group and one of the treated group had died. In ten weeks the experiment was terminated, and at this time there were 61.6 per cent (sixteen) of the treated animals alive and 33.3 per cent (seven) of the untreated controls. In addition it should be pointed out that in ten of the animals in the treated group no lesions could be found either grossly or microscopically at post-mortem examination. Lesions were found in all of the control animals (untreated) with the exception of one animal which died an accidental death by drowning soon after the experiment began.

The clinical appearance of the rats which were being treated showed a remarkable difference from those in the control group. The latter group developed lassitude and somnolence and appeared chronically ill, whereas the treated group showed few or no signs of illness.

In summary, forty-seven animals (rats) were inoculated with *Torula histolytica*, twenty-one served as a control group and twenty-six served as a treated group. Treatment consisted of 3,000 units of streptomycin given subcutaneously in normal saline in divided doses daily. Sixty-one per cent of the treated animals and thirty-three per cent of the untreated animals survived. In ten of the treated group no lesions could be found at post-mortem examination. In only one of the untreated did we fail to find the lesions.

#### CONCLUSION

In this experiment streptomycin seems to have exerted a beneficial effect upon experimental torulosis. It is not known whether its effect is due to the streptomycin or to some additional factor present in its market preparation.

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## NUTRITION AND EXPERIMENTAL DIABETES

### I THE DIABETIC RESPONSE OF WEANING RATS TO INTRAVENOUS DOSES OF ALLOXAN

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THE induction of diabetes in small animals by subcutaneous or intraperitoneal injection of solutions of alloxan monohydrate is an uncertain procedure. The wide variations in response with these routes of administration are in part related to the rapid inactivation of alloxan by body fluids. In the course of work involving the use of large numbers of young diabetic rats we found it necessary to investigate the relationship of the intravenous dose of alloxan to (1) the selective destruction of pancreatic cells which will lead to diabetes without concomitant injury to other visceral organs, notably the liver and kidneys (the validity of metabolic studies in animals made diabetic with alloxan is dependent upon this selective anatomical alteration), (2) the incidence of diabetic response among injected animals and (3) the extent and permanency of the defect of carbohydrate metabolism produced.

#### EXPERIMENTAL

Young albino rats of the Hisaw or Sherman strains were used. We could detect no strain difference in response to alloxan. The animals were maintained on dog food\* before injection. All animals ranged between 30 and 50 grams in weight at the time of injection. A 3 per cent solution of alloxan monohydrate† was prepared in sterile distilled water immediately before each injection. It was found that the available preparations of alloxan monohydrate are often variable in appearance and solubility. The preparations used therefore were assayed chemically by the manometric procedure of Archibald<sup>1</sup> and the dosage was adjusted to the amount of alloxan in the sample. The solution was drawn into a 0.5 ml. tuberculin type of syringe equipped with a one half inch No. 26 needle. The animal was placed in a mailing carton with the tail protruding through a hole in the center of a cork stopper used to close the carton. The container was conveniently clamped to a ring stand. Cleaning the tail surface with a soapy cloth followed by a brisk rub with a dry cloth will adequately dilate the four tail veins without heating. A tourniquet is not necessary.

The needle is introduced, bevel up directly over the vein and on the distal one third of the tail. Veins over the proximal third although apparently larger, are injected with difficulty. The vein is closely attached to the skin,

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\*Gaines Dog Meal Formula A-13H.  
†Eastman Kodak Company, Rochester, N. Y.

so the needle must be inserted at a small angle with the tail axis. When blood appeared at the syringe nipple, the calculated volume of alloxan solution was injected quickly. When undue resistance or perivascular blanching occurred indicating extravasation, the animal was discarded. With this procedure one can soon learn to inject about twenty-five rats per hour.

The animals were placed in group cages and at intervals were placed in individual metabolism cages for twenty-four hours. In young rats several criteria may be used for determining the presence of diabetes. The data below are based upon twenty-four-hour urine glucose excretion as measured by a photometric modification of the method of Somogyi<sup>2</sup> and blood sugar determinations by the method of Reinecke<sup>3</sup> after a preliminary five-hour fast. Failure to gain weight, polydipsia, polyuria, and, after several weeks, cataract formation are also manifestations of diabetes. A fasting blood sugar above 150 mg per cent or twenty-four-hour urine glucose excretion of more than 0.1 Gm was considered an indication of diabetes.

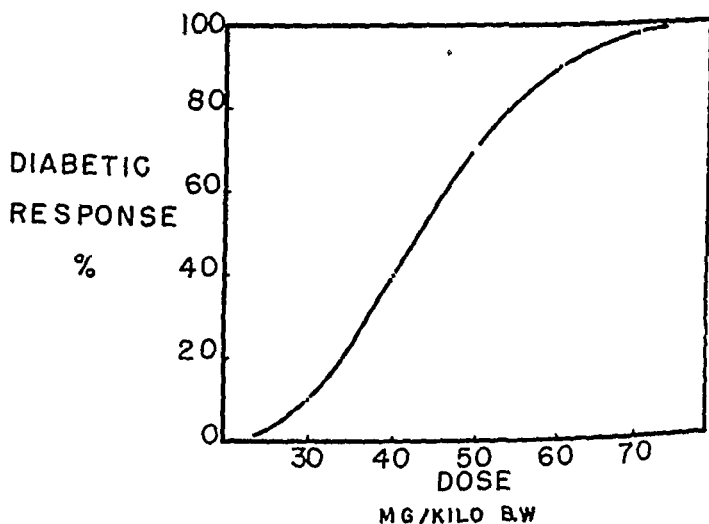


Fig 1—The forty-eight hour diabetic response of weanling rats to the intravenous injection of alloxan monohydrate

Fig 1 represents the typical forty-eight-hour response of several groups of animals to the various doses of alloxan used. The incidence of diabetes thereafter is to some extent determined by the nature of the diet fed. In general the dog food diet was found to give the best survival. When a purified diet containing 18 per cent protein was used, 100 per cent mortality was obtained in ten weeks contrasting with 100 per cent survival in a similar group of animals maintained on the dog food diet. Table I illustrates the effect of certain diet modifications upon the course of the diabetes, with mortality as a criterion of dietary adequacy.

The groups of rats in Table I were composed of animals with comparable severity of diabetes as judged by the criteria listed. They were placed upon the various diets five days after the injection of alloxan. It will be noted that al

TABLE I THE EFFECT OF DIET UPON SURVIVAL IN YOUNG DIABETIC RATS (ALLOXAN)

DIET	NUMBER OF RATS	SURVIVORS				TOTAL MORTALITY (%)
		DAYS				
		14	28	56	70	
Dog food	8	8	8	8	8	0
10% Protein	7	5	3	2	2	72
20% Protein	8	8	7	4	2	75
40% Protein	8	7	6	4	2	75
40% Protein plus 1% NaCl in water	7	6	5	4	2	72
18% Protein purified	20	14	11	6	0	100

Gaines Dog Meal \ 13H

though the mortality at seventy days was approximately the same with various levels of protein, the early mortality was higher with the lower protein levels. The survivors at seventy days represent the animals with mild diabetes. In the various diets the protein was increased at the expense of carbohydrate. The inadequacy of the purified diet, as judged by mortality is striking when compared with either the dog food diet or the semipurified diets (containing 10 per cent brewers' yeast as a source of B vitamins). Addition of 1 per cent sodium chloride did not significantly alter the survival.

The influence of diet upon the severity of diabetes and the survival of the diabetic animals will be the subject of a later publication.

## DISCUSSION

The immediate mortality (about 7 per cent) in animals receiving intravenously 70 mg of alloxan per kilogram of body weight has been caused by injury of the liver and kidneys of these animals. The susceptibility of animals to this extrapancreatic toxicity of alloxan appears to be as variable as diabetic susceptibility. Doses above 70 mg per kilogram lead to increasing incidence of this complication.

Selection of the optimum dose is thus determined by two parameters. First doses below 40 mg per kilogram give diabetic responses in so few animals that many animals and much time are lost. Second doses above 70 mg per kilogram often lead to injury of organs other than the pancreas thus complicating later metabolic studies.

The 60 mg per kilogram dose level has been found most practical. The 10 to 20 per cent of nonresponding animals can be used as alloxan controls in many experiments. Such controls may in part answer the criticism that alloxan may act as a nonspecific cellular toxin leading to complications other than diabetes. Thus in our work it has been useful to control each experiment with animals not injected with alloxan and with alloxan injected nondiabetic animals. Although we have not studied these latter control animals with glucose tolerance tests, the twenty-four urine glucose excretion is in effect a tolerance test and would be expected to reveal animals with minimal diabetes.

Histologic studies of animals receiving alloxan have revealed no evidence of renal or hepatic damage attributable to the drug in doses of 60 mg per kilogram of body weight or less. Animals receiving higher doses and dying within forty-eight hours frequently have shown such lesions. Of thirty animals receiving 70

mg per kilogram which survived the injection forty eight hours, only one animal later showed cirrhotic changes in the liver with ascites, and this after a period of fourteen months. This was believed to be a late result of mild hepatic injury by alloxan.

Consideration of the severity of diabetes among the responding animals indicates that with increasing doses the number with severe diabetes increases. This would seem to indicate a range of sensitivity among animals extending from the few animals which respond with severe diabetes through the majority which respond moderately to the few who will not respond at all. Experience with repeated injections of those animals that do not respond indicates that they often are resistant even to 80 to 100 mg per kilogram of alloxan.

It should be emphasized that young rats made diabetic with alloxan offer an extremely sensitive experimental tool for study of nutritional relationships in the course of diabetes. Measurements of body weight, water consumption, or urine excretion offer a simple means of assaying the influence of experimental procedures.

#### SUMMARY

The diabetic response of weanling rats to intravenous doses of alloxan has been studied.

A procedure has been described which will allow production of alloxan diabetes in the majority of injected animals with minimal occurrence of extra pancreatic alloxan injury.

An experimental plan has been suggested which will allow more adequate control of experiments by utilization of nonresponding alloxan-injected animals as additional control animals.

The course of animals made diabetic with alloxan and surviving more than forty-eight hours is determined in part by diet. Survival is greatest in animals fed a diet of dog food and poorest in animals on a highly purified diet. Higher levels of protein and decreased amounts of carbohydrate favored survival on the purified diets.

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## MYELITIS FOLLOWING THE ADMINISTRATION OF NEOARSPHENAMINE

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ONE of the hazards involved in the therapeutic use of arsenicals is the danger of central nervous system reactions. These accidents although rare are serious, causing approximately one half the deaths from arsenical therapy.<sup>1</sup> In a survey of 496 253 protocols, one death due to central nervous system reaction occurred for every 5,398 persons treated and every 28 768 injections given. In a group of 158 patients with central nervous system reactions the mortality rate was found to be 76 per cent.

These reactions include encephalitis, encephalomyelitis and myelitis. Encephalitis is by far the most common. It has been widely described and the usual picture of headache, fever, and vomiting followed by convulsions and coma is well known. Encephalomyelitis, a more rare complication is less widely described. The signs of cerebral involvement usually precede and may mask the evidences of cord lesions. About half these patients have been considered to have encephalitis alone until post mortem examination showed unsuspected pathologic changes in the spinal cord. Because myelitis is uncommonly reported and infrequently considered, the following protocol is presented.

A 20 year old woman was given two intravenous injections of neoarsphenamine by the family physician because she suspected she had been exposed to syphilis. A man with whom the patient had been in intimate contact had been discovered recently to have a secondary syphilitic rash. The patient had no signs of syphilis and a serologic test for syphilis was negative. Nevertheless it was decided that he should be treated. Accordingly the patient was given 0.3 Gm. of neoarsphenamine intravenously without untoward reaction. Seven days later he was given 0.45 Gm. intravenously.

Two hours after the second injection the patient had a severe shaking chill followed by a fever of 39.5 C. and moderately severe malaise. She went to bed and for two days had recurrent chills and fever and anorexia. On the first day the physician gave her 600 000 units of procaine penicillin. On the morning of the second day a faint pink nonpruritic rash of small irregular macules appeared on the face, arms and neck. The patient also noted that her throat was lightly sore and that her eyes burned. The evening of the second day following the second injection of neoarsphenamine the rash faded and the chills and fever subsided but the malaise increased. The patient noticed then for the first time that her feet felt numb and that she had difficulty in walking. She was barely able to walk with assistance to the bathroom and void.

In the twelve hours that followed a painless sensation of numbness crept up the patient's legs to the trunk, arms and neck. As this occurred all voluntary motion was lost in the legs which lay limply on the bed. The patient also developed urinary retention. She was mentally clear and had no pain. There was no difficulty in talking, swallowing or breathing and no difficulty with vision. When the sensory defect was at its maximum the patient noted transient impairment of voluntary motion in the arms and hands. The sensation of numbness

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receded from the neck, arms, and upper chest as rapidly as it had appeared, hence on the following morning when she was seen by the physician, a sensory level was apparent above the navel. At this time (the third day following the last injection of neocarsphenamine) there was a low grade fever, malaise was gone, and appetite was returning. Complaints were sore throat, loss of sensation in trunk and legs, inability to move the legs, and urinary retention.

The patient remained at home in bed three more days and did not consider herself seriously ill although she had a slight fever of about  $38^{\circ}\text{C}$  and was bothered by her sore throat. The sensory level did not change nor did motor function in the legs improve. The patient could sit without support after being helped to a sitting position. She voided spontaneously, slowly, at long intervals (sixteen to twenty hours), passing large amounts of urine. On the sixth day frequency and urgency developed quite suddenly, with voiding every twenty to thirty minutes. Distressed by this and discouraged by the lack of improvement in the function of her legs she came to the New York Hospital.

On admission the patient appeared moderately ill but was alert, cooperative, and oriented. Temperature was  $37.5^{\circ}\text{C}$ . There were minimal but generalized lymph node enlargement, mild conjunctivitis, and pharyngitis. A single, small, faintly pink, irregular macule was present on the left cheek. The remainder of the general physical examination was not contributory. Examination of motor and sensory functions showed no abnormalities of the head or neck. There was no weakness or wasting in the arms. Rapid, rhythmic, alternating motion was slightly impaired in the right arm, and the biceps, supinator, and pronator reflexes were slightly more active than on the left. Sensory perception was normal in the neck, arms, and trunk above the level of D 8. From D 8 to D 10 there was dyesthesia to pin prick, light touch, and temperature testing. Below D 10 there was anesthesia to these modalities but vibration and position sense were intact above and below this level. There was no sweat level or pain on percussion over the spine. Superficial abdominal reflexes were absent. The patient could sit without support if assisted to the upright position. The legs were symmetric and without wasting, but were completely flaccid. The only voluntary motion possible in the legs was a slight degree of flexion in the right knee. The tendon reflexes were normal but slightly more brisk in the right leg. Plantar responses were extensor. There were mass withdrawal responses in the legs. Rectal sphincter tone was normal but the perianal region was anesthetic. The patient was able to void spontaneously but was forced to urinate every twenty to thirty minutes in order to escape incontinence of urine.

On admission, urine analysis showed only an occasional white cell. Repeated analysis showed no albumin or red cells. The blood urea nitrogen was 11 mg per 100 milliliter. The Mazzoni reaction was negative and remained so. Blood counts were within normal limits. Heterophile agglutinations were negative on admission and ten days thereafter. Culture from the throat showed a mixed flora with no predominating organism. A patch test with neocarsphenamine on the seventh hospital day produced a 2 cm indurated erythematous area in sixty hours. Lumbar puncture on admission gave crystal clear fluid with no evidence of block or increased pressure. Repeated cerebral spinal fluid cultures and Wassermann reaction were negative. Cerebral spinal fluid sugar and chloride determinations were within normal limits. The results of repeated lumbar punctures were as follows:

DAY	LYMPHOCYTES	POLYMORPHONUCLEARS	PROTEIN
1	3	0	55
2	4	1	39
7	6	2	24
13	5	0	30

There was slow but steady improvement which began with a slight regression of the level of anesthesia on the first hospital day. On the second hospital day the patient disclosed that she had received two injections of neocarsphenamine. Because of previous reports of myelitis following neocarsphenamine injections, it was decided to begin British anti-lewisite (BAL) therapy. A total of 1,300 mg of BAL was given over a four day period. By the eighteenth hospital day anesthesia was confined to a few spotty areas on the lower legs and in the perianal region. These changes persisted and in the entire area which was

formerly anesthetic there was dysesthesia to pin, light touch and temperature testing similar to that found in the area from D 8 to D 10 on admission. The patient gradually regained voluntary motion and strength in the legs but improvement was retarded by the transient appearance of a moderate degree of exten or spasm. The reflex abnormalities in the arms and legs did not change with the exception of the plantar reflex which became flexor. The patient was able to walk without support on the thirty seventh hospital day. She continued to have frequency of urination but the interval had increased to one to two hour.

Most standard texts do not list arsenical myelitis as one of the toxic manifestations of arsphenamine therapy. So few cases have been reported that it is impossible to draw inferences as to cause and effect as has been done with arsenicals and encephalitis. In all, there are only nine cases similar to that of the patient herein described.<sup>3</sup> The chance occurrence of myelitis of unknown cause in a patient receiving arsenicals must be considered. The case reports are so few that no clear cut clinical picture is typical. However many of the reports are strikingly similar. Scott and Reinhart<sup>4</sup> describe a patient in whom the onset and progression of symptoms were almost identical and followed the second injection of neoarsphenamine. This patient however did not survive. Many patients have been suspected of having had Herxheimer reactions and only one previous patient did not have syphilis. However in the few that have been autopsied the histopathology of the cord was similar to that in the brains of patients with encephalitis and in the cords and brains of those with encephalomyelitis.

In the New York Hospital case the possibility of a Herxheimer reaction may be waived. The possibility of intercurrent myelitis of other cause remains and cannot be eliminated.

BAL has been shown by Stocken and co workers to protect rats from lethal doses of therapeutic arsenical compounds.<sup>5</sup> Eagle and Magnuson<sup>6</sup> have reported fifty five cases of arsenical encephalitis treated with BAL. The overall mortality rate was 11 per cent as opposed to 76 per cent of an earlier untreated series. Severe cases with convulsions, coma or both were observed to respond dramatically in twenty four to seventy two hours particularly if BAL was given early. There is now a convincing body of evidence that the toxic effects of arsenicals are due primarily to the fact that they combine with and block physiologic enzyme systems vital to the cellular economy. The antidotal action of BAL is referable to its ability to remove arsenicals from combination with these enzyme systems in the cells. There has been discussion as to whether the central nervous system reactions from arsenicals particularly myelitis are due to toxicity or sensitivity. The fact that BAL has been dramatically effective in encephalitis suggests that the mechanism here involves toxicity, reversible with early BAL therapy. Since the histopathology is similar in encephalitis and myelitis, it can be postulated that myelitis is a toxic reaction which should respond just as dramatically.

In the New York Hospital case BAL was given too late to allow inferences concerning therapeutic or diagnostic values. Yet the outcome here is unusual since only two of the previously reported patients survived one with severe residuals. If myelitis following the injection of neoarsphenamine is due to toxic effects of this agent it should respond to BAL when given early. It would be one variety of myelitis for which there is a specific therapeutic agent.

## SUMMARY

Myelitis following the administration of neoarsphenamine is a rare but serious complication of arsenical therapy. It is suggested that, if arsenical compounds have been given to a patient developing myelitis, BAL therapy be instituted.

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# LABORATORY METHODS

## RECOVERY AND ESTIMATION OF RADIOACTIVE ISOTOPES FROM BIOLOGIC TISSUES

I GOLD

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IN ORDER to study the distribution of gold in animal and human tissues it was necessary to develop a rapid and quantitative method for the recovery and estimation of this element. Toward this end use was made of the experience gained, during 1942 and later, with the electrodeposition methods employed on the Manhattan Project (unpublished papers<sup>1, 2</sup>). Investigations along these lines also were encouraged by the successful use of electrodeposition in the determination of radioactive iron by Ross and Chapin<sup>3</sup>, Hahn<sup>4</sup> and others.

By the use of the electrodeposition technique thin uniform films of the radioelement may be obtained which then can be used for the determination of radioactivity. Standard Geiger or alpha counters are employed for these measurements as the case may be.

As early as 1918, DeWitt, Caldwell, and Leavell had shown that it was possible to determine gravimetrically the amounts of gold present in biologic tissues after electrodeposition on platinum electrodes. Details of the method employed by these authors could not be found although a brief description was furnished, namely, Kjeldahl digestion, followed by evaporation to about 1 cc, treatment with aqua regia and ammonia, and neutralization with HCl. The solution was filtered, made slightly acid with HCl and buffered with phosphoric acid sodium phosphate. Electrodeposition was carried out in 40 cc of solution at 60°C and 1 to 12 volts. Current density was not given. Recoveries of 80 to 100 per cent were obtained with 0.02 to 0.3 mg of gold.

Scott<sup>5</sup> gives methods for the determination of gold in electrodeplate baths and also outlines a microanalytical procedure for all using platinum anodes and cathodes and potassium cyanide plating solutions. Procedures for the commercial plating of gold are given in standard references and trade journals<sup>6, 7</sup>. Most of these methods employ gold anodes and either chloride or cyanide plating baths.

In general, the method herein described follows closely the standard procedures for the separation and electrodeposition of gold.

From the Division of Medical Physics, University of California.

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† 43.

‡ 518.

§ Pages 199-210.

## EXPERIMENTAL

*Electrodeposition Apparatus*—The anode consists of 0.040 inch diameter platinum wire wound in a flat spiral. The vertical length of wire is sealed into glass and both are connected into a suitably drilled brass rod  $\frac{3}{8}$  inch in diameter. The latter is fixed in the chuck of a stimer rotating at about 300 revolutions per minute.

Cathodes are  $1\frac{1}{2}$  inch diameter disks, 0.002 inch thick, either of platinum or gold-plated copper. Platinum must be used for exacting work, but for reasons of economy the plated copper was substituted in carrying out these experiments.

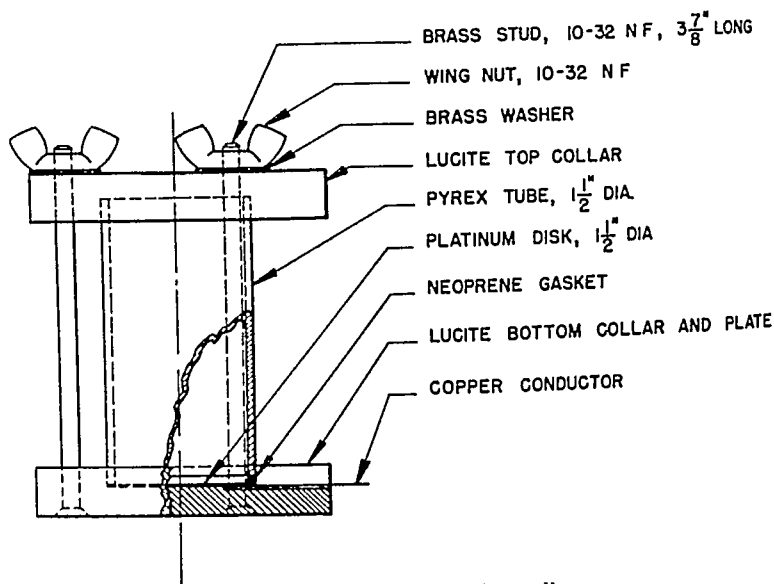


Fig. 1—Electrodeposition cell

The electrodeposition cell<sup>\*</sup> shown in Fig. 1 uses a  $1\frac{1}{2}$  inch outside diameter Pyrex tube, 3 inches long.

Operating current is supplied from two cells of a 6-volt storage battery. An ammeter with a range of 0 to 1 ampere and a 1-watt, 20 ohm rheostat are placed in series with the electrodeposition cell. The actual apparatus† consists of twenty-four such units arranged in two banks of twelve each. Each unit has a toggle switch for throwing the ammeter in or out of the circuit. For work with gold, a better arrangement would be to use an ammeter with a scale of 0 to 0.2 amp and a variable resistance of 50 ohms. Each unit should have a fuse to protect both the ammeter\* and the resistance, and a voltmeter should be provided to measure the potential drop between anode and cathode. The apparatus used for this work had been built for another purpose and could not be modified in the time available.

*Procedure*—The weighed tissues are digested in 25 ml Erlenmeyer flasks, using either aqua regia or concentrated nitric acid plus superoxol. In either

\*Detailed drawings can be furnished upon request.

†Designed and constructed by Mr. Robert Loevinger of this laboratory.

case, only small amounts of reagent are added at one time. If excessive frothing is encountered 1 drop of octyl alcohol is added. Complete oxidation of the latter, as well as the tissue, must be accomplished before proceeding further.

As a carrier, 20 ml of a reagent grade gold chloride solution may be added either before, during or after digestion, although addition at an early stage in the digestion is recommended. The concentration of this solution should be accurately known, and the solution should contain approximately 5 mg per milliliter of metallic gold as the chloride. The concentration of gold in the carrier solution is determined either by electrodeposition on tared platinum disks or by evaporating a known volume to dryness in a tared porcelain crucible followed by heating to decompose the chloride, cooling and weighing.

Upon completion of digestion, excess nitric acid is removed by adding successive small amounts of concentrated HCl with heating. The resulting solution is evaporated to 1 to 2 ml and transferred to a 15 ml centrifuge cone. Only a roughly quantitative transfer is needed since the electrodeposited film is to be weighed, and recoveries as low as 90 per cent or even 80 per cent are acceptable.

The separation of the gold from all or nearly all of the associated elements in the tissue digest is accomplished by reducing the gold to the metallic state. The reducing solution is made up to contain approximately 10 per cent  $\text{NH}_4\text{OH}$  HCl and 0.4 per cent  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . Two milliliters of a freshly prepared solution are used for each 10 mg or less of metallic gold. Reduction is hastened by placing the cones in a beaker of hot water which is kept just below the boiling point for fifteen to thirty minutes. The ferrous sulfate is omitted from the reducing solution when urine and feces samples are to be run.

After centrifugation, the supernatant liquid is discarded. The centrifuge cone wall and the gold may be washed and centrifuged and the wash solution also discarded. Although this is not considered necessary for routine work, it was carried through when the set of determinations shown in Table I was made.

The pellet of precipitated gold is readily dissolved in a mixture of 4 drops of hydrochloric acid plus 1 drop of nitric acid. Placing the cone in a beaker of hot water accelerates the reaction which is usually complete within fifteen minutes.

The aqua regia solution of gold is made all at once with 5 ml of 1N sodium hydroxide. After mixing, this solution is transferred to the electrodeposition cell. The latter should be assembled with a tared and numbered disk and should have 2 ml of a 5 per cent solution of potassium cyanide in 0.1N sodium hydroxide covering the disk before the gold solution is added. The centrifuge cone is washed with distilled water, which is also poured into the cell. In routine work this is accomplished by filling the cone once with distilled water, followed by transfer to the cell.

The anode is placed in the solution at about  $\frac{3}{4}$  to 1 inch from the cathode. The current is adjusted to approximately 85 milliamperes. Since the actual plating area is about 8.5 sq cm, the current density will be 10 Ma per square centimeter, or approximately twice the current recommended for commercial electroplating.\*

Deposition is allowed to proceed for one hour at room temperature. At the end of this time an additional 2 ml of the cyanide solution are added, followed by another hour of electrodeposition.

Upon removal of the anode the cell contents are discarded, and the cell is washed with distilled water. After disassembly the disk is again washed, rinsed in acetone, and placed in a warm place to dry. The gross weight is generally obtained before the radioactivity is determined, although this is not essential.

### *Notes on the Analytic Procedure —*

**Digestion** As often happens in the wet digestion of biologic tissues, there are occasional samples which precipitate sparingly soluble crystalline salts upon evaporation to small volume. When such occurred during this work, the solution and precipitate were transferred together to the centrifuge cone and the procedure was carried through as with the other samples. The presence of this material, which was always in very small amounts, apparently did not interfere with the results. To insure that no radioactive gold is lost, however, any insoluble material should be dissolved at some point between the time the carrier is added and the time reduction is completed, even though an extraneous precipitate appears later as it frequently does with urine and feces samples. This precipitate does not interfere with the electrodeposition. When octyl alcohol is used to prevent frothing, a waxy material is frequently formed which has a low melting point and which may not be observed until the solution is cooled to below room temperature. Precautions should be taken to obtain complete digestion of this organic matter.

**Reduction** The use of both hydroxylamine and ferrous sulfate was investigated. The latter reduced the gold more rapidly than the former, and there was usually a small amount of unwetted gold floating on the meniscus after centrifuging. This gold was either lost upon decantation or had to be removed on a stirring rod and returned later. Reduction with hydroxylamine occurred in three distinct phases, namely, reduction to aurous, formation of colloidal gold, and finally, precipitation of the metal. Unwetted gold was not usually obtained when this reagent was employed, but the formation of a light gold mirror was observed frequently. When a mixture of both reductants was used, both phenomena were less pronounced, and the rate of reduction was about the same as with ferrous sulfate alone. The influence of acidity and salt concentration upon the rate of reduction and the character of precipitated gold was not investigated. A considerable range of conditions was encountered, but the results in all cases were satisfactory.

**Solution of Gold in Aqua Regia** When mirror formation was encountered, the cone was placed in only about 1 inch of hot water to hasten the dissolving reaction. Sufficient aqua regia condensed on the wall of the cone to dissolve the mirror completely.

**Addition of NaOH** If insoluble salts are present after digestion, a white precipitate is sometimes present or is formed at this point. If desired, this insoluble material may be removed by centrifuging before the solution is transferred to the electrodeposition cell. This was not done with the experiments reported.

**The Cathode Disk** Both platinum and gold plated copper disks may be cleaned by immersion in warm concentrated sulfuric acid dichromate cleaning solution. With the latter type of disk, immersion should not be for longer than two to three minutes, since the plate does not offer sufficient protection to the copper to prevent its dissolving. Both types of disk must be thoroughly rinsed with water after this treatment. The platinum disks may be dried directly after washing but an acetone rinse is recommended first for the gold plated copper. Heating the platinum disks over a burner in order to dry them is not recommended, since they then lose their temper and are more difficult to handle without bending. Early experiments carried out by Bertrand<sup>10</sup> indicated that plain copper disks lost rather than gained weight during electrodeposition. Without investigating this problem further, it was decided to try gold plated copper disks in the hope that the formation of oxide film on the surface of the copper and attack of the copper by the cyanide plating solution could thus



be reduced or eliminated. The alternative was to use either gold or platinum disks, which in view of the number needed and the difficulties involved in removing the radioactive layer would not be economical. While the use of plated copper did not entirely eliminate the weighing error, the results obtained were satisfactory for this type of analysis. The thickness of the commercially made gold plate was not determined. The actual plating was done on strips of copper  $1\frac{1}{4}$  by  $1\frac{3}{4}$  by 0.002 inch. The  $1\frac{1}{4}$  inch disks were then punched out.

**Electrodeposition.** It was frequently observed that the plated disks in the assembled cells would discolor upon transference of the gold solutions to them. This occurrence was minimized but not entirely eliminated, by adding the cyanide solution to either the cone or the electrodeposition cell before transference. The addition of the KCN directly to the cone prevents precipitation of auric hydroxide, the latter sometimes occur upon allowing the solution to stand for several hours with NaOH alone. Neither the cause of the discoloration reaction nor its influence upon the gravimetric determination was investigated. The color of the gold plate on platinum disks was either yellow or light orange while that on gold plated copper varied from light rose to brown. The color in the latter case is pointed out by Weisburg and Graham\* may be due to the presence of copper (from the cathode) in the plating solution. Gold plates on platinum frequently showed small blisters in which case the deposit was easily broken loose by scratching. The use of a wetting agent such as one of the alkyl aryl sulfonates as suggested by Hartshorn<sup>11</sup> might eliminate this difficulty. Blistering was not observed with the plated copper disks but the deposit on these disks could be rubbed off to an appreciable extent perhaps because of the admixture of copper, hence highly active disks must be handled carefully in order to prevent contamination of counters, and so on, due to powdering off of small unweighable amounts of gold. The decision to use alkaline cyanide plating solutions rather than acid chloride was based upon the assumption that in the case of the latter there probably would be some attack of the platinum anode due to the formation of free chlorine and therefore deposition of platinum with the gold. The possibility of attack on the platinum by the cyanide however is not ruled out.

#### ANALYTIC RESULTS

The data shown in Table I should be divided into three groups. Determinations 77 through 84 were carried out with what is considered to be the proper amount of carrier gold to insure complete recovery of the radioactivity and at the same time to give a sufficient amount of deposit for gravimetric purpose, namely 10 milligrams. Determinations 85 through 92 were carried out with less than 10 mg of carrier gold. With both of these groups, the various solutions were added directly to the assembled electrodeposition cells as follows: 5 ml of 1N sodium hydroxide, 4 drops of hydrochloric acid, 1 drop of nitric acid, 2 ml of 5 per cent potassium cyanide, the carrier gold solution and the radioactive gold solution. Distilled water was added in each case to make a total volume of approximately 30 milliliters. Determinations 77 through 92 were actually eight sets of duplicates.

The third group comprises Determinations 93 through 96. This group of four identical determinations was carried out to test the recovery and electroplating procedures under carefully controlled conditions. For this purpose three normal adult mice weighing 20 to 25 grams each were dry ashed in the muffle furnace at 600° C. The ash was taken up in aqua regia and the excess nitric acid removed as outlined under Procedure. The resulting solution was diluted to 150 ml and 10 ml portions were used for each of these four determinations. The carrier and radioactive gold solutions were added and the foregoing procedure was then followed.

TABLE I RECOVERY OF RADIOACTIVE GOLD FROM SOLUTIONS CONTAINING VARYING AMOUNTS OF RADIOACTIVITY AND CARRIER

DETERMINATION	NORMAL GOLD ADDED (MG)	RADIO GOLD ADDED (ML)	INCREASE IN DISK WEIGHT (MG)	RECOVERY OF NORMAL GOLD (%)	COUNTING DATA			EXTRAPOLATED COUNT SHELF 2 (TIME = 0)	RECOVERY OF RADIOACTIVITY (%)
					COUNTS PER ML $Au^{198}$ (PER MIN)	COUNTER SHELF	ELAPSED TIME (HR)		
77	10.3	1	10.3	100	340	4	2	2400	-
78	10.3	1	10.5	102	330	4	2	2350	-
79	10.3	2	10.5	102	1610	2	36	2400	-
80	10.3	2	10.4	101	1585	2	36	2350	-
81	10.3	5	10.2	99	330	4	2	2350	-
82	10.3	5	10.4	101	335	4	2	2400	-
83	10.3	10	10.4	101	330	4	3	2350	-
84	10.3	10	10.4	101	320	4	3	2300	-
85	0	2	-0.3	-	1480	2	35	2150*	91
86	0	2	-0.4	-	1480	2	35	2150*	91
87	1.0	2	1.0	100*	1400	2	45	2300*	98
88	1.0	2	0.9	90*	1400	2	45	2300*	98
89	2.1	2	1.9	90*	1385	2	45	2250*	96
90	2.1	2	2.1	100*	1435	2	45	2350*	100
91	5.1+	2	5.1	99	1450	2	45	2350	-
92	5.1+	2	5.2	101	1435	2	45	2350	-
93	10.3	2	10.6	103	1450	2	46	2350	100
94	10.3	2	10.5	102	1435	2	46	2350	100
95	10.3	2	10.6	103	1435	2	46	2350	100
96	10.3	2	10.5	102	1400	2	46	2300	98
Average				101				2350	

Count on shelf 4 x 69 (approx) = count on shelf 2

\*Not included in the averages

†Based upon 2350 counts per milliliter per minute

Half life of  $Au^{198}$  2.7 days

TABLE II RECOVERY OF GOLD FROM ASHED BIOLOGIC TISSUE SOLUTIONS, 98 MG OF CARRIER GOLD ADDED

ARTHRITIC PATIENT					RABBIT			
TISSUE SAMPLE	SAMPLE WT OR VOL	AU <sup>198</sup> RECOVERY			TISSUE SAMPLE	SAMPLE WT (GM)	AU <sup>198</sup> RECOVERY	
		MG	%				MG	%
Synovia	0.33 Gm	9.2	94		Kidney	5.0	9.6	98
Muscle	0.71 Gm	9.8	100		Spleen	2.0	9.7	99
Superficial fasciae and fat	1.22 Gm	9.7	99		Testicle	2.8	9.7	99
Skin	0.31 Gm	9.4	96		Articular cortex	2.6	9.6	98
Deep fascia	0.31 Gm	9.8	100		Articular cortex	3.0	9.8	100
Blood	2 MI	9.8	100		Adrenal	0.2	9.7	99
Blood	2 MI	9.7	99		Liver	6.2	9.5	97
Blood	2 MI	8.0	82		Marrow	1.2	7.9	81*
Blood	2 MI	9.4	96		Lung	3.3	10.0	102
Blood	2 MI	9.5	97		Aorta	0.3	9.3	95
Blood	2 MI	9.1	93		Synovia	0.2	9.6	98
Blood	2 MI	9.3	95		Synovia	0.4	9.5	97
Blood	2 MI	9.3	95		Tendon	0.2	9.4	96
Blood	2 MI	9.1	93		Tendon	0.2	9.4	96
Synovial fluid	1.5 MI	8.8	90		Small intestine	2.8	9.6	98
Second day urine	100 MI	9.0	92		Bone	1.0	9.6	100
First day feces	Total†	8.5	87		Heart	3.1	9.8	100
Second day feces	Total†	9.4	96		Muscle	4.1	9.5	97
					Skin	0.8	9.8	100
Average				94				

\*Electrodeposition cells leaked values not included in the average

†Weight not obtained

Two typical sets of biologic data are given in Table II. These data were collected during the course of the work with arthritis carried out by Bertrand and co workers<sup>12</sup>

### DISCUSSION

No explanation is offered for the 101 per cent average recovery of added gold, Table I. Since the error involved in biologic work is usually greater than the error which would be introduced by using the per cent carrier gold recovery as a factor in calculating the radiogold recovery, no further work was carried out to eliminate this discrepancy. The per cent recovery values shown in Table II therefore were used in calculating the original tissue gold activity<sup>1</sup> not reported herein.

One surprising result obtained as shown in Table I (Determinations 85 and 86), was the 91 per cent recovery of radioactive gold when no carrier gold was added to the solution in the electrodeposition cell. The solution of radioactive gold contained 5  $\mu$ g of metallic gold per milliliter in the form of sodium gold thiosulfate. The quantitative recovery of gold during electrodeposition therefore was greater than one would be led to expect the losses in disk weight notwithstanding.

In carrying out the gold purification procedure with tissue digests from the arthritic patient ferrous sulfate was used as a reductant. Trouble was experienced only with the urine and feces samples. Upon adding the reductant to the digested solutions from these samples a red brown flocculent precipitate was obtained in three of four cases. The fourth case appeared to give a normal precipitate, but unfortunately the sample was lost upon centrifuging and a better observation could not be made. The abnormal precipitates were completely soluble in concentrated HCl showing that reduction had not occurred. The addition of hydroxylamine to these samples gave normal precipitation of the gold. The use of the mixed reducing solution with these two types of sample is not recommended, hydroxylamine alone should be used.

The results with the rabbit tissue samples also shown in Table II were obtained using hydroxylamine alone as a reductant. All determinations given in Table II were carried out using routine or roughly quantitative transfer techniques.

### SUMMARY

A method is described for the recovery and estimation of radioactive gold from biologic tissues.

The results obtained with this procedure are well within the allowable error for this type of determination.

Under a rather wide range of conditions satisfactory results were obtained in all cases.

The method is rapid requiring no special techniques and the electrodeposition equipment is fairly simple.

The author is indebted to Dr J H Lawrence, Dr C A Tobias and Dr J J Bertrand for assistance and advice and to Jean Luce for the digestion of the many biologic samples needed for this work.

The radioactive gold samples at first were supplied through the courtesy of J G Hamilton and later by the Atomic Energy Commission

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## THE USE OF PRESERVED ERYTHROCYTES FOR THE DETECTION AND IDENTIFICATION OF Rh ANTIBODIES

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AN IMPORTANT requisite for any Rh testing laboratory is a constant supply of group O human erythrocytes of various Rh types. For the qualitative detection of Rh antibodies, cells of types CDE (Rh<sub>1</sub>Rh<sub>2</sub>) and cde (Rh negative) are highly desirable while for accurate identification of the antibodies which are encountered, cells of Cde (Rh') cDe (Rh<sub>2</sub>) and cdE (Rh') and occasionally other rare types must be available. Even the largest blood typing centers frequently have difficulty obtaining donors of the desired types however and to the smaller laboratory this difficulty may constitute an insurmountable handicap to adequate antibody testing.

We have attempted to solve this problem by preserving blood specimens in anticoagulant solutions. We have found as has been reported by others that when blood is mixed with an appropriate volume of sterile Alsever's or a c.d. solution and is stored in the refrigerator it keeps with a minimum of hemolysis for many weeks. In evaluating such preserved blood for our purposes there were two questions which demanded an answer: (1) Do the cells retain their agglutinability? (2) Do they retain their specificity? In a preliminary attempt to answer these questions the following study was made:

A donor group A MN Rh<sub>1</sub>rh (cDE/cde) was bled aseptically and 5 cc of the blood were mixed in each of a series of capped bottles with 5 cc of sterile Alsever's solution. One bottle was withheld for immediate study and the remainder were stored at refrigerator temperature (4°C). At weekly intervals one bottle was removed from the refrigerator for study and discarded.

Each specimen was titrated against a standard anti-D (Rh<sub>2</sub>) blocking serum of proved keeping qualities in the following manner:

- (1) An approximately 2 to 3 per cent suspension of cells was made in 30 per cent bovine albumin† by adding 2 or 3 drops of the blood anticoagulant mixture to 1 cc of albumin.
- (2) The standard anti-D (Rh<sub>2</sub>) serum was serially diluted in normal AB serum and 1 drop of each dilution was placed in a series of small agglutination tubes.
- (3) To each tube was added 1 drop of the cell suspension.
- (4) All tubes were incubated in a 37°C water bath, centrifuged three minutes at moderate speed and examined under the dissecting microscope for agglutination which was recorded as 1 plus to four plus.

The titers obtained are recorded in Table I. It may be seen that there was only very little fluctuation in titer, probably due entirely to variations in technique until the seventh week. At that time there began a definite decrease in titer which was proved by adequate controls not to be due to deterioration of the serum. It was at this time that the stored blood first showed gross hemolysis.

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‡Prepared specifically for Rh testing by the Armour Laboratories, Chicago, Ill.

TABLE I TITER OF STANDARD ANTI Rh<sub>0</sub> (D) SERUM AGAINST Rh rh (cDE/cde) CELLS STORED IN ELSEVER'S SOLUTION AT 4° C

AGE OF BLOOD (WK)	TUBES OF SERIAL DILUTION							TITER
	1	2	3	4	5	6	7	
0	4	4	4	3	2	1	-	32
1	4	3	3	2	1	1	-	32
2	4	4	3	2	1	-	-	16
3	4	4	3	3	2	1	-	32
4	4	4	3	2	1	-	-	16
5	4	4	3	2	1	-	-	16
6	4	4	3	2	1	-	-	16
7*	4	4	3	2	-	-	-	8
8*	4	2	-	-	-	-	-	2
9*	3	2	1	-	-	-	-	4
Control†	4	4	3	2	1	-	-	16

\*Grossly hemolyzed

†Fresh cells of same donor tested against stored serum at same time as 9 week old cells

A saline cell suspension of each specimen was likewise tested against the high-titered human anti-A and anti-B sera which are routinely used in this laboratory for Landsteiner grouping as well as against the routine Rh subtyping sera. In every case the reactions were exactly as they were when the blood was fresh. No false agglutination or false negative reactions occurred.

Similar observations have been made since in blood specimens of all of the Landsteiner types and a wide variety of Rh and H<sub>1</sub> types. In the two years that Elsever's and a c d solutions have been used in our laboratories, there has never been any evidence that cells preserved in this way are not perfectly reliable for Rh testing as long as there is no gross hemolysis. This period has varied in our hands between two and eight weeks, depending upon the care with which contamination is avoided and the amount of agitation to which the cells are subjected.

#### DISCUSSION

The advantage of a satisfactory preserving medium for blood cells for Rh testing is perfectly obvious. Since the introduction of the Elsever technique into our laboratories we have had little difficulty in keeping ourselves stocked with cells of all of the Rh subtypes which we have needed in our work. It is simple to keep a roster of patients and donors of various types, and at periods of a few weeks or a month to call in the appropriate individuals for renewal of the store of preserved bloods. We have found it most satisfactory to divide each donation between two or more bottles of anticoagulant, so that if one becomes contaminated or hemolyzed in handling, the supply of cells will not be lost entirely. When cells of some particular type are needed for testing, a few drops are drawn aseptically from the appropriate bottle and a cell suspension is made up in saline or albumin. If one is using a technique such as the slide test of Diamond and Abelson or the conglutination test of Wiener in which the presence of an aqueous solution of sodium citrate might interfere with the test, it is necessary to centrifuge the mixture, pour off the supernatant, and resuspend the cells in undiluted serum or plasma.

We feel that this method of preserving blood has become an indispensable adjunct to the proper functioning of our blood grouping laboratory

#### SUMMARY

Alsever's and a c d solutions have been found valuable for preserving whole blood of various antigenic types. Cell suspensions made from such preserved blood are satisfactory for testing sera for the corresponding Rh and Hr antibodies.

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## METHODS TO INCREASE ACCURACY IN THE USE OF HAYEM'S SOLUTION FOR RED BLOOD COUNTS

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IN 1934 Bryan and Garrey<sup>1</sup> published a description of a rotor for the mechanical mixing of blood in diluting pipettes which affords more exact white counts than other methods of mixing. The erythrocytes, however, often were found to ball when diluted with the usual diluent for red blood counts, Hayem's solution. This effect was found to be due to the bichloride in the diluent, and Toison's fluid was therefore recommended for red counts.

Ch'u and Forkner<sup>2</sup> observed clumping of erythrocytes with the use of Hayem's solution in certain pathologic conditions even when the pipettes were shaken by hand, and they suggested substitution of Gower's solution to avoid the presence of bichloride.

Neither of these suggestions, as is also true of other substitutions that have been offered over the years, has received popular adoption. The reasons for this are several: either the diluents suggested were impractical for the general run of cases and laboratories, or the precautions offered in the use of them, as well as of Hayem's solution, proved inadequate upon wider usage, or custom on the part of the medical profession at large and the relative degree of satisfaction that has been afforded by the use of Hayem's solution determined that that should remain the diluent of choice for red counts.

Hayem<sup>3</sup> devised the formula which is now in common use as a diluent for red counts at a time when blood counts were beginning. His methods of obtaining and mixing blood, and even of superimposing it on the chamber for counting, were less refined than those employed today, and the demands for accuracy were correspondingly different. Nonetheless, use of the fluid has sufficiently stood the test of time to be continued regardless of the dissenting voices from period to period. As Ch'u and Forkner<sup>2</sup> have enumerated, the ideal diluent should preserve the cells and sharpen their visibility without danger of destroying any of them or of too greatly distorting them, should keep without deterioration, should eliminate danger of growth of organisms, and should permit satisfactory distribution of cells in the pipette and on the counting chamber. Hayem's solution fulfills all the criteria outlined for an ideal diluent except the indispensable one of dependably satisfactory distribution of cells. With mixing by hand, or with vigorous mechanical shaking, errors due to unsatisfactory distribution can usually be reduced, though not eliminated. With the advent of the mechanical rotor which was proved so satisfactory for white blood counts, which can rotate as many as eight pipettes simultaneously, and is therefore desirable as a time-saving device for clinical and research laboratories

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and which is now manufactured commercially the inequalities of distribution of erythrocytes in Hayem's solution have become more evident and more in need of remedy

In view of the fact that the suggestions already made for substitution of other diluents for red counts have failed to be adopted it seemed preferable to attempt modification of Hayem's solution in such a manner as to eliminate the dangers to distribution inherent in it, rather than to encourage further use of different diluents. Such modification has been attained in one of two ways either by reduction of the proportion of bichloride to as low as that recommended by Jorgensen,<sup>4</sup> together with careful control of the pH of the diluent between 5.0 and 7.0, or by addition of such colloids as gelatin or lecithin to Hayem's or Jorgensen's solution without concern for pH below 7.0. Chu and Forkner found Jorgensen's solution unsatisfactory in the cases that they studied, but as the importance of the hydrogen ion concentration in the use of Jorgensen's solution was not recognized at that time it is probable that failure to control that factor featured in their findings.

The gentleness of the movements of the rotor of Bryan and Garrey served as a tool to show that the danger of clumping with Hayem's solution often is masked by other methods of mixing, but is actually inherent in the use of bichloride in diluents irrespective of the manner of mixing. The rotor permitted analysis of the causes that underlie the clumping and that feature in the remedies which assure against it. These studies are given in detail in a companion paper. This report will be confined to the practical points in the preparation and use of the modifications of Hayem's solution that have been found to prevent clumping, and to comparison between red blood counts made with Hayem's solution and with the modified diluents.

#### METHODS

The formula for Jorgensen's<sup>4</sup> solution is as follows:

Na <sub>2</sub> SO <sub>4</sub>	2.5 Gm
NaCl	0.5 Gm
HgCl <sub>2</sub>	0.05 Gm
H <sub>2</sub> O (distilled)	100 cc

The formula for Hayem's solution modified by the addition of gelatin is as follows:

Na <sub>2</sub> SO <sub>4</sub>	2.5 Gm
NaCl	0.5 Gm
HgCl <sub>2</sub>	0.25 Gm
Gelatin	0.01 Gm
H <sub>2</sub> O (distilled)	100 cc

The gelatin used was a powdered preparation. Other preparations were not tried but it is judged that any preparation of sufficient purity to rule out the presence of harmful electrolytes would be satisfactory. It should of course, be kept dry for weighing.

Preparation of Hayem's solution containing gelatin is made most easily by dissolving (1) the desired amounts of sodium sulfate, sodium chloride and gelatin in one half the de-

<sup>4</sup>Sold as Bacto Gelatin by Difco Laboratories, Detroit, Mich.

sired amount of water, warming if necessary to hasten solution of the gelatin, and (2) the desired amount of bichloride in the other half of the desired amount of water. When the two solutions are complete they should be mixed, with precautions to avoid foaming, and stored. Where large amounts of the diluent are desirable because of frequency of demand, it is helpful to make a sufficient supply of the double strength bichloride solution and to mix this with fresh batches of the double strength saline gelatin mixture as desired. The double strength bichloride solution keeps indefinitely in Pyrex glass and may be filtered, if necessary, before mixture with the solution containing gelatin. The mixed solution must not be filtered, but merely decanted, should the precipitate occur that is common in saline solutions of bichloride.

Both of the modified diluents should be kept in Pyrex containers since solution of the glass inevitably carries the danger of elevation of the pH to the point where clumping of the red cells cannot be prevented. Furthermore, even readjustment of the pH—if it has become elevated by solution from a container—is not sufficient to avoid clumping. This indicates, of course, that electrolytes dissolved from glass must have effects upon clumping in addition to those produced by increase in the pH\*. It has been determined that either of the modified diluents can remain in contact with the particular types of diluting pipettes that were used for these experiments for as long as twenty four hours without change in pH.

Red counts made with the modified diluents were compared with counts made with the standard Hayem's solution. The counts were carried out under experimental conditions that were planned to simulate conditions that might occur in routine clinical use. Judgment as to satisfactory distribution for blood counts was made, first and foremost, by microscopic inspection of the filled counting chambers. The counts were carried out merely for experimental assurance that they would, in reality, meet the usual standards for red blood counts. They were not planned primarily for a comparison on a statistical basis, and although the number of counts with any given diluent is considerable, the number for any given condition with that diluent is admittedly too few for satisfactory statistical analysis. When compared on that basis, nevertheless, the groups of counts with each of the diluents do show trends that are in agreement with the conclusions based on the microscopic studies. Therefore the significant data from the counts are presented in Table I and Fig 1, and are discussed as probable statistical support of conclusions that depend primarily upon microscopic studies. As additional security a larger series of counts made with Hayem's solution alone and with Hayem's solution containing gelatin was obtained for a single experimental condition. This material is presented in the discussion of the experimental data.

The blood for all of the pipettes for any given series of counts was drawn at the same time from a freely flowing finger stick. The same subject and observer were used throughout. Both Haak and Trenner automatic pipettes were used in the comparison. No differences due to the model of pipette were found. The pipettes always were given ten quick shakes by hand immediately after filling. The mixing of the blood with diluent was carried out by different methods which were, in turn, employed for different periods of time. When the blood was mixed by rotation, a commercial model of the Bryan Garrey rotor was used. It was tested against one of the original experimental models and was found to give identical results. When the blood was mixed by a mechanical shaker, an experimental model devised by Beard† was employed. In some instances mixing was started at once. In others, the pipettes were allowed to stand for various intervals before mixing was started. These data for the different sets of counts are given in Table I. Levy-Hauser counting chambers with the improved Neubauer ruling were used throughout. Twenty or more drops always were expelled from the pipettes before the chambers were filled. When successive drops were to be counted from a single pipette, ten or more drops also were

\*I wish to thank Dr. Ann M. Mot Department of Biochemistry Vanderbilt Medical School Nashville, Tenn. for these determinations and for the helpful interest which initiated investigation of the causes which underlie the clumping of blood with Hayem's solution.

†The model was made while Dr. Beard was connected with Vanderbilt Medical School Nashville, Tenn. Similar models have been used in numerous laboratories through the courtesy of that school.

expelled between each filling and all chambers were filled in sequence. Those not to be counted at once were allowed to stand on moist paper under a bell jar.

There was a striking tendency for the red cells to become aligned with the lines of the chamber and to leave the centers of the small squares relatively depleted of cells when the diluent contained gelatin and the chamber was allowed to stand for any considerable time after filling. Since analysis of the data to follow indicates a gain in accuracy in

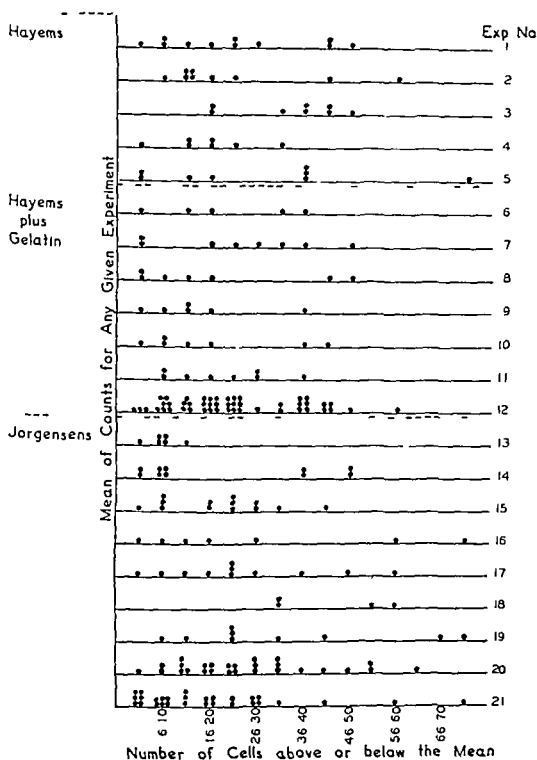


Fig. 1—Scatter chart showing the number of counts in a series that fall within any given deviation of the mean of the series. Experimental conditions can be obtained by reference to Table I.

counts made in the presence of gelatin, this alignment of the red cells would seem to be unimportant to the accuracy of the counts and to represent simply a mutual shift in position of cells that had been well distributed, rather than a basic fault in distribution.

In conformity with the usual clinical routine five small squares of the counting chamber that is, one fifth of a square millimeter, were included for each count. The values are

sired amount of water, warming if necessary to hasten solution of the gelatin, and (2) the desired amount of bichloride in the other half of the desired amount of water. When the two solutions are complete they should be mixed, with precautions to avoid foaming, and stored. Where large amounts of the diluent are desirable because of frequency of demand, it is helpful to make a sufficient supply of the double strength bichloride solution and to mix this with fresh batches of the double strength saline gelatin mixture as desired. The double strength bichloride solution keeps indefinitely in Pyrex glass and may be filtered, if necessary, before mixture with the solution containing gelatin. The mixed solution must not be filtered, but merely decanted, should the precipitate occur that is common in saline solutions of bichloride.

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†The model was made while Dr. Beard was connected with Vanderbilt Medical School, Nashville, Tenn. Similar models have been used in numerous laboratories through the courtesy of that school.

cells was observed in Jørgensen's solution with normal blood when the values for pH were below 5.0. With values above that lysis was not detected in normal blood nor in a patient with congenital hemolytic icterus in relapse.\* The saline fragility of this patient at the time of the observations was above 0.56, the red count, 2,560,000, the reticulocyte count, 12.8 per cent, and the icteric index, 15 units. The erythrocyte counts of this patient made from pipettes diluted with Jørgensen's solution and allowed to stand for as long as twenty-four hours before counting did not differ from those from similarly filled pipettes that were counted immediately nor from pipettes that were diluted with Hayem's solution containing gelatin. Lysis must be detected by observation of fading cells or through comparison of counts made by methods in which there is no question of lysis, since the effect of bichloride upon any liberated hemoglobin conceals the reddening of the suspension which ordinarily announces hemolysis.

Berkson and co-workers\* do not state specifically what diluent they employed in their series of statistical studies concerning the limits of significance in red blood counts, but they do state that they used the methods in common practice. It is therefore assumed that they used Hayem's solution. Since the clumping that has been found to be an underlying tendency in the use of this diluent may often be so slight as to escape attention it is reasonable to suppose that the values which these authors obtained are greater than would be the case had they used one of the modifications of Hayem's solution discussed in the present studies. This assumption is strengthened by the counts made in these studies. It will be seen from the table that when Hayem's solution alone was used, the standard deviations were at times as great as those found by Berkson and co-workers, namely 37.5 counted erythrocytes (375,000 total red blood cells). In the counts with Hayem's solution containing gelatin or with Jørgensen's solution under controlled conditions the values are decidedly lower.

#### CONCLUSIONS

Hayem's solution carries an inherent capacity for the balling or clumping of cells when used for red blood counts. The tendency toward clumping is magnified by gentle rotation but underlies all manners of mixing. It affects the dependability and accuracy of counts made with Hayem's solution.

The capacity for clumping inherent in Hayem's solution is due to the component of bichloride. It can be eliminated by decrease of the proportion of bichloride to that of Jørgensen's solution, together with control of the pH of the diluent between 5.0 and 7.0 or by addition of gelatin to Hayem's solution without concern for pH below 7.0.

Counts made with these modifications of Hayem's solution are subject to less deviation than are counts made with Hayem's solution alone under the same conditions.

Case History No. 11,383, Vanderbilt Hospital, Nashville, Tenn. I am very grateful to Dr. Edgar Jones and Dr. Henry Warden, Department of Medicine, Vanderbilt Medical School, Nashville, Tenn., for the clinical data concerning this patient and for allowing me to make these studies. I am also very grateful to the Department of Social Service of the Vanderbilt Hospital for the interest which they exercised in helping me to contact this patient.

Use of Hayem's solution containing gelatin allows greater flexibility of experimental conditions without loss of protective power than does use of Jorgensen's solution. Addition of gelatin to Hayem's solution is, therefore, the modification preferred.

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## A SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF URINARY BILIRUBIN

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A REVIEW of the literature on bile pigment metabolism and tests of hepatic function reveals that many quantitative tests for urinary bilirubin have been proposed. Probably the most satisfactory to date is that devised by Goodson and Sheard<sup>1</sup> in 1940 employing diazobenzenesulfonic acid. In 1941 Scott employed the diazo reagent of Ehrlich and the unit system of van den Bergh in the quantitative determination of urinary bilirubin. His method is the most accurate, being sensitive to 0.02 mg of bilirubin per 100 ml of urine. It is, however, a procedure too difficult and time consuming to be employed as a routine clinical laboratory test. Gellis and Stokes<sup>2</sup> employed a modification of the methylene blue test of Franke<sup>3</sup> in their studies of infectious hepatitis in 1945. This was at best only a roughly quantitative test. Stokes and co workers<sup>4</sup> in 1946 attempted to adapt the methylene blue test to the spectrophotometer but with little success, principally because the color produced in the methylene blue test is not the color of a true solution but a combination of colors. The barium strip modification of Harrison's spot test was developed by Watson and Hawkinson<sup>5</sup> in 1946 as a semiquantitative test.

It becomes immediately evident that a simple yet reasonably accurate, quantitative test of bilirubinuria is needed. The value of such a test in the diagnosis and prognosis of hepatic and prehepatic hyperbilirubinemia and in the diagnosis of hepatitis in the preicteric stage is obvious. In order to meet this need the following method is elaborated. It is based on the oxidation of bilirubin to a green derivative and the estimation of the latter spectrophotometrically at a wave length of 670 millmicrons.

### METHOD

In this procedure a modified Fouchet's reagent is utilized as an oxidizing agent. This is prepared with 6.25 Gm of trichloroacetic acid and 2 ml of a 10 per cent ferric chloride solution diluted to 200 ml with distilled water. A Coleman Junior spectrophotometer is used with 15 mm cuvettes and adapter. A standard bilirubin solution is prepared by dissolving 10 mg of pure bilirubin in 100 ml of chloroform. To 20 ml of this standard solution are added 80 ml of 95 per cent ethyl alcohol resulting in a 2 mg per cent solution of bilirubin. Further dilutions of the latter solution are made as desired with 95 per cent ethyl alcohol. Into a 15 mm cuvette are placed 4 ml of the chloroform alcohol solution of bilirubin of known concentration, 4 ml of 95 per cent ethyl alcohol and 2 ml of the modified Fouchet's reagent. A reference tube is prepared by adding to 4 ml of 20 per cent chloroform alcohol solution 4 ml of 95 per cent ethyl alcohol and 2 ml of 3.125 per cent trichloroacetic acid. Ten minutes are allowed to elapse after the addition of the modified Fouchet's reagent to permit the full development of the green color. The spectrophotometer is set at a wave length of 660  $\mu$  and at 100 per cent transmission with the reference tube. A reading then is taken

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with the bilirubin solution and plotted on semilogarithmic graph paper using per cent transmission as the abscissa and concentration of bilirubin in milligrams per 100 ml as the ordinate. The point is then connected by a straight line to the point—concentration equals 0, transmission equals 100 per cent. The Lambert Beer Law,  $c = -K \log T$ , will be expressed by the curve represented by this straight line (Fig 1). From this curve the concentration of any similarly oxidized bilirubin solution can be determined if the percentage of transmission is known.

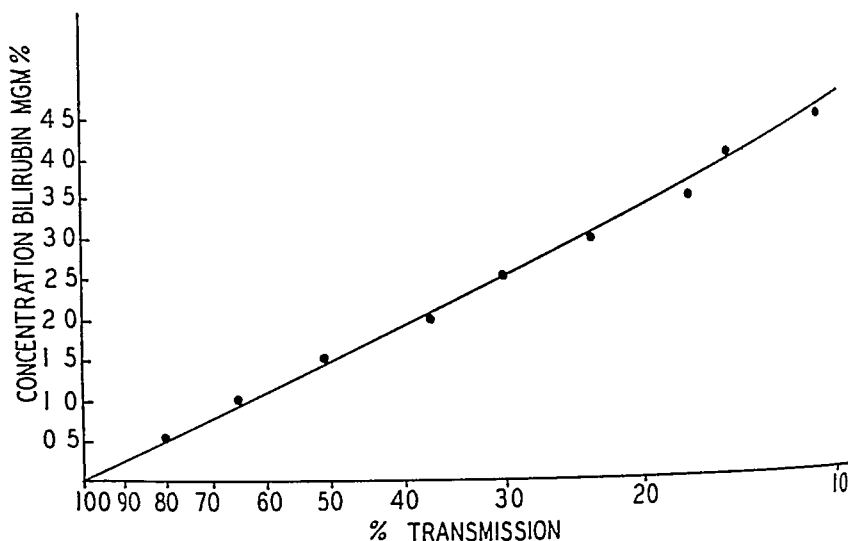


Fig 1

The wave length of 670  $m\mu$  was chosen after the preparation of a spectrophotometric curve using a 2 mg per cent alcohol chloroform solution of bilirubin treated with the reagent as described. The reference was prepared with 8 ml of 20 per cent chloroform alcohol solution plus 2 ml of modified Fouchet's reagent. This curve was plotted from readings made at 25  $m\mu$  intervals from 400 through 700 millimicrons. From this curve it was evident that the ideal wave length was from 650 to 700 millimicrons. After readings were made at 5  $m\mu$  intervals from 650 to 700  $m\mu$ , it was found that a wave length of 670  $m\mu$  was most desirable.

#### PROCEDURE FOR DETERMINING URINARY BILIRUBIN

To 4 ml of undiluted urine in a 15 mm cuvette are added 4 ml of 95 per cent ethyl alcohol and 2 ml of the modified Fouchet's reagent. A reference tube is prepared with 4 ml of urine plus 4 ml of 95 per cent alcohol and 2 ml of 3.125 per cent trichloroacetic acid. After ten minutes a reading is taken in the spectrophotometer at a wave length of 670  $m\mu$ , and the concentration of bilirubin is determined from the previously prepared graph.

#### DISCUSSION

This procedure provides a simple quantitative method for the determination of urinary bilirubin. Attempts at recovery of bilirubin added to the urine were highly successful as can be seen in Table I. The bilirubin was added to the urine in the form of icteric serum, the concentration of which was determined spectrophotometrically by the method of Malloy and Evelyn. The data provided by these determinations serve to confirm the validity of the curve prepared in the foregoing.



TABLE I VARIATION AND PER CENT RECOVERY BY SPECTROPHOTOMETRIC METHOD OF BILIRUBIN ADDED TO URINE

CONCENTRATION OF BILIRUBIN (MG/ML)	RECOVERY OF BILIRUBIN (MG/ML)	VARIATION (MG/ML)	RECOVERY (%)
1.30	1.25	0.05	96
1.65	1.50	0.15	91
0.93	0.90	0.03	97
1.05	1.50	0.10	90
0.83	0.80	-0.03	96
0.90	0.85	0.05	94
1.20	1.15	-0.05	96
0.50	0.50	0.00	100
0.60	0.60	-0.00	100
0.70	0.65	-0.10	93
0.85	0.75	-0.10	88
2.45	2.35	-0.19	96
2.85	2.65	0.15	93
3.00	2.85	-0.10	95
3.50	3.40	0.15	97
4.05	3.90	0.05	96
5.00	4.95	0.10	99
5.55	5.45	0.00	98
6.00	6.00	0.15	100
6.50	6.35	0.02	98
1.70	1.68	0.02	99
0.16	0.14	0.02	87
0.77	0.75	0.02	97
0.39	0.37	0.06	95
1.00	0.94	0.09	94
1.50	1.41	0.05	94
Mean		0.07	95

The original Fouchet's reagent will yield a green color if used in this procedure. This, however, has a pH of 1 and it was found that a 25 per cent trichloroacetic acid solution (pH 1) if used as a reference also would yield a green color. When the pH of the reagent and reference solution was brought up to 4, no oxidation of the bilirubin to a green derivative took place in the reference tube. The oxidation at this pH apparently is dependent on the ferric chloride.

By using an equal volume of urine in the reference tube any extraneous color resulting from other chromogens is compensated for. The alcohol in no way enhances or interferes with the production of the green color. It has been introduced to free any bilirubin globin that might be present in a proteinuria.

The normal urinary bilirubin concentration has not been studied adequately, but the preliminary studies on random samples of urine of normal patients done in this laboratory agree with those of Naumann<sup>8</sup> who found it to be less than 0.5 mg per cent.

To date, salicylates are the only substances found to interfere with this test. If present in sufficient concentration they will produce a pink color when the reagent is added.

#### SUMMARY

A simple and accurate spectrophotometric method for the quantitative determination of urinary bilirubin has been presented. This test is dependent on the production of a green color resulting from the oxidation of bilirubin with an acid ferric chloride reagent. The choice of optimal wave length and the preparation of a standard bilirubin solution are discussed.

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# A SCHOLANDER MICROMETER BURETTE OF SIMPLE CONSTRUCTION

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THE Scholander micrometer burette has been of value for the precise measurement of the delivery of small volumes of liquid. The original design<sup>1, 2</sup> specified a micrometer the anvil of which had to be annealed

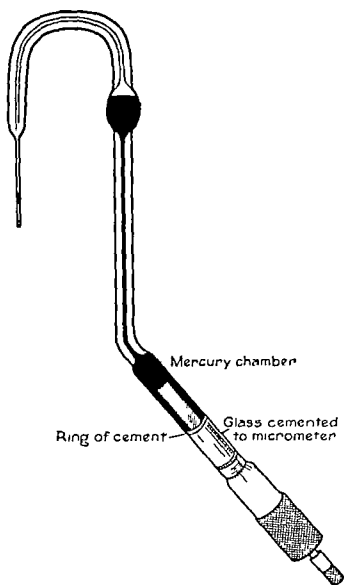


Fig 1

drilled, and threaded. The use of a micrometer head and a minor rearrangement in design eliminates all machine work and reduces glass blowing to a minimum. The result is a simple apparatus easily constructed by anyone with a knowledge of glass blowing at a fraction of the usual cost.

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The micrometer head used is a Brown & Sharpe No 294 RS. The glass part is made of 1 mm Pyrex capillary tubing and standard 11 mm Pyrex tubing. The latter has a normal internal diameter of 9 mm with a variation of 0.5 millimeter. In order to obtain a close fit between the 11 mm tubing and the micrometer head, the internal diameter of the tubing should be smaller than the shoulder to be inserted. This diameter is then enlarged to the proper size by grinding it internally with emery paper wrapped around a wooden dowel rod until a close fit is obtained. Small variations in diameter can be taken care of by the cementing process.

In order to obtain a satisfactory cemented seal, both parts should be heated to about  $145^{\circ}\text{C}$ , preferably in a thermostatic oven. The inside of the lower end of the mercury chamber is coated with de Khotinsky cement, which will melt at the temperature given. The shoulder of the micrometer head then is fully inserted into the mercury chamber. If this is done properly, a continuous ring of cement will form at the end of the micrometer, effectively sealing off from the mercury chamber any air spaces which may have formed between the metal and the glass.

The seal must be carefully inspected after the apparatus has cooled. Should the ring of cement not be continuous, the defect can be corrected by introducing a small piece of the cement into the mercury chamber through the micrometer head at the proper place and then reheating the apparatus.

The other details of construction will be apparent from the drawing and need no further description.

We have found that heavy grease in the spindle, as suggested by Scholander, occasionally traps air bubbles which are forced into the mercury chamber by the movement of the spindle. We use a small amount of light machine oil to lubricate the micrometer threads. The hydrostatic head of mercury above the chamber effectively prevents air from entering along the spindle. It is essential to maintain this head at 5 cm or more. The glass tip of the burette should be short enough to avoid a siphoning effect.

The burette described delivers volumes as accurately as the original model of Scholander.

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## A MODIFIED TECHNIQUE FOR THE EXAMINATION OF BODY FLUIDS

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IN SOME instances a diagnosis of malignant tumor can be made by examining the cellular content of centrifuged sediment from body fluids but the technical difficulty of obtaining a maximal amount of sediment has limited the usefulness of these studies. Schenken and McCoid<sup>1</sup> suggested the use of collodion bags suspended in glass centrifuge tubes. Marcuse and Coulter<sup>2</sup> reported

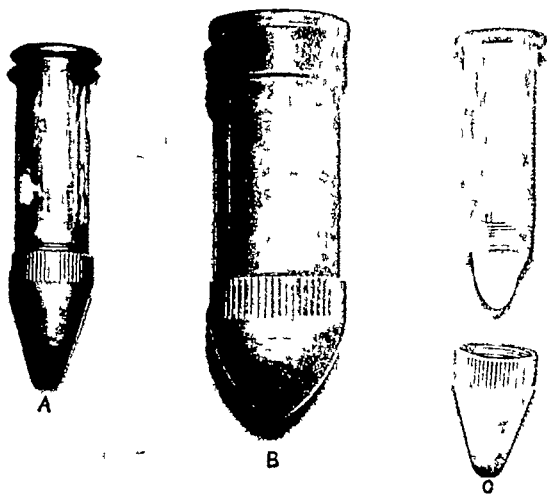


Fig. 1—Metal centrifuge tubes used to aid in the study of body fluids. A and B show the intact tubes of two sizes. C illustrates the metal tip removed and the appearance of the collodion bag within the tube.

the use of a metal centrifuge tube made up of three separate parts which simplifies the removal of the sediment. The technique described requires twenty-four to thirty-six hours for completion of the examination. It was decided to combine the advantages of these two devices in an effort to make the study of body fluids more simple and rapid.

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The bottoms were removed from two sets of standard size metal centrifuge tubes and threaded to receive detachable tips. Each tube of the smaller set holds 15 c.c. and each of the larger set accommodates 50 c.c. (see Fig. 1).

In preparation for use, the tip is first detached and a thin film of stopcock grease applied to the inner surface of the tip and to the lower two inches of the inner surface of the barrel. The tip is screwed tightly to the barrel and 2 or 3 c.c. of moderately thick collodion is poured into the tube. With the tube held horizontally and slowly rotated, the entire inner surface is evenly coated with collodion. The excess is drained from the tube and the collodion is partially dried to form a stable film. It is advisable to determine by experience the necessary thickness of the collodion membrane.

The specimen is poured into the tubes and centrifuged at 1,500 revolutions per minute for fifteen to twenty minutes. The detachable metal tip is then slowly unscrewed, leaving the intact collodion sac containing the sediment suspended from the barrel of the tube. A small incision just above the level of the sediment will allow the supernatant fluid to drain with a minimum of turbulence. The collodion tip is then cut off and dropped into Bouin's solution or formalin. After fixation for at least one hour, the block of sediment can be dehydrated, embedded in paraffin, and sectioned like tissue.

If desired, the sediment can be smeared on clean glass slides, fixed immediately in a 1:1 mixture of 95 per cent alcohol and ether, and stained by the Papanicolaou technique. In these cases the collodion tip is removed, inverted on a finger, and the inner surface rubbed on a slide. Even when no sediment is visible, a fair concentration of the cells often can be seen on the smear.

This technique has been especially fruitful in the study of urinary tract lesions, since renal tumors have been detected by examination of small volume washings of cells from the kidney pelvis. It also has been used to concentrate the cells in pleural and ascitic fluids, secretions aspirated from the bronchial tree, and gastric washings.

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\*If the volume of body fluid is too great to be accommodated by the metal tubes it can be centrifuged in large glass containers and most of the supernatant fluid decanted. The liquid containing the sediment then can be transferred to the special tubes.

## STUDIES ON THE DESTRUCTION OF RED BLOOD CELLS

VI THE SPLEEN AS A SOURCE OF A SUBSTANCE CAUSING AGGLUTINATION OF THE RED BLOOD CELLS OF CERTAIN PATIENTS WITH ACQUIRED HEMOLYTIC JAUNDICE BY AN ANTIHUMAN SERUM RABBIT SERUM (COOMBS SERUM)

PHILIP F. WAGLEY, M.D., SHU CHU SHEN, M.D., FRANK H. GARDNER, M.D.,  
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### INTRODUCTION

BOORMAN, Dodd, and Loutit<sup>1</sup> have demonstrated that the immune serum prepared by Coombs, Mourant, and Race<sup>2</sup> by the injection of human serum (not red cells) into rabbits may be used to distinguish certain types of acquired hemolytic jaundice from congenital hemolytic jaundice. They observed that the washed red blood cells from five patients with acquired hemolytic jaundice were agglutinated by this serum. On the other hand the washed red blood cells from seventeen patients with congenital hemolytic jaundice were not agglutinated. The test was originally developed by Coombs and associates<sup>3</sup> for the detection of the incomplete Rh agglutinin on the supposition that such sensitized red blood cells might carry adsorbed antibody globulins. The red blood cells are agglutinated presumably as a physical manifestation of the union of the globulin or other substance on the surface of the red cells with the antibody in the test serum.

Acquired hemolytic jaundice has been reported as an independent acute or chronic entity<sup>4, 5</sup> or in association with malaria, tuberculosis, cirrhosis of the liver, Hodgkin's disease, leukemia, carcinomatosis, and cysts and tumors of the ovary.<sup>6, 7</sup> Splenomegaly is often present in such conditions, and splenectomy in some instances, although not necessarily altering the cause of the disease, may be beneficial by decreasing red blood cell destruction. In some cases the agglutination titer of the patient's red blood cells in antihuman serum rabbit serum declines after splenectomy.<sup>8</sup> Such observations also encountered in our own experience, have raised the question of whether the spleen, which contains both lymphocytes and reticulo endothelial cells as potential sources of antibodies, might be a source of the substance presumably adhering to the red blood cells of these patients. The principal objective of the experiments reported here was to examine the spleens removed from patients with acquired hemolytic jaundice and various other conditions for the presence of substances possessing an affinity for normal red blood cells.

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## METHODS

All spleens were obtained at operation and unless immediately employed in the experimental procedure were frozen at  $-4^{\circ}\text{C}$  and preserved at that temperature. Spleens from three patients with acquired hemolytic jaundice and from two patients with thromboerythrocytopenic purpura were studied immediately after splenectomy. Spleens from one patient with acquired hemolytic jaundice, three with congenital hemolytic jaundice, two with thromboerythrocytopenic purpura, three with congestive splenomegaly (Banti's syndrome), and two with Gaucher's disease were studied after being frozen for from one to two years.

A portion of each spleen was finely ground with a domestic meat chopper. The ground pulp obtained from the spleens that were studied immediately after splenectomy was then stirred vigorously with approximately twice its volume of distilled water. The distilled water was then allowed to drain briefly from the pulp through gauze. This procedure was repeated three times, at which point the pulp usually contained no microscopically visible intact red blood cells. Then the splenic pulp was resuspended in normal saline solution which was finally removed by draining through gauze. The pulp was then weighed. In the case of the frozen spleens the effect of the previous freezing and thawing served to destroy the red blood cells present and so rendered treatment with distilled water unnecessary. The absence of red blood cells from the pulp of these spleens was confirmed in each instance by inspection of a small sample under the microscope.

A sample of pulp from each spleen was then incubated for from one to two hours at  $37^{\circ}\text{C}$  with 3 cc of a 40 per cent suspension of either normal Group O Rh negative red blood cells or of red blood cells of the patient's blood group. The red blood cells had previously been carefully washed three times with physiologic salt solution. The amount of splenic pulp employed in each incubation was judged by the amount that was shown not to cause significant hemolysis of the washed red blood cells during a preliminary period of incubation of one hour at  $37^{\circ}\text{C}$ . With the fresh spleens, 12 Gm of pulp were employed. With the previously frozen and thawed spleens, from 15 to 12 Gm of pulp were used. After the incubation period, two or three times the volume of physiologic salt solution was added to the mixture which was then stirred and filtered through gauze one or more times in order to separate the red blood cells from the splenic pulp. The red blood cells obtained were then carefully washed three times with normal physiologic salt solution and finally made up into a 5 per cent suspension in that medium. A control sample of red blood cells was treated in a similar fashion except that it was not added to splenic pulp.

The test for the presence of adsorbed substance was carried out by incubating for one hour at  $37^{\circ}\text{C}$  0.2 cc of the 5 per cent suspension of red blood cells with 0.2 cc amounts of serial dilutions in physiologic salt solution of an antihuman serum rabbit serum. The rabbit serum was prepared according to the method of Coombs, Mourant, and Race.<sup>2</sup> Its strength was determined by a modification of the method of Haberman and Hills against a suspension in physiologic salt solution of Rh positive red blood cells which had previously been sensitized with an Rh blocking antibody with a titer of 1:64. The rabbit serum caused microscopic agglutination of the washed cells sensitized with the blocking antibody in a dilution of 1:128.\*

At the conclusion of the incubation period each red blood cell suspension was studied microscopically for evidence of agglutination. The control sample of red blood cells was handled in the same fashion except that its agglutinability was determined only with the undiluted test serum. In addition, tests were made of the agglutinating power of the antihuman serum rabbit serum against washed red blood cells derived from the peripheral blood and from the splenic pulp of the four patients with acquired hemolytic jaundice at the time of splenectomy.

## RESULTS

Inspection of the data shown in Table I indicates that subsequent to incubation with the splenic pulp from the four patients with acquired hemolytic jaundice, normal washed red blood cells, either Group O Rh negative or belonging

\*It is essential that the serum employed in testing the effects of incubation with splenic pulp has a relatively high titer.



to the same blood group as that of the patient, were agglutinated by the anti human serum rabbit serum. Though definite when observed under the microscope, in no instance was the agglutination present when the serum used was diluted. On the other hand, normal red blood cells after similar incubation with the splenic pulp from patients with congenital hemolytic jaundice were

those derived from the peripheral blood of the same patients. Not shown in the tables is the fact that the red blood cells from the splenic pulp and peripheral blood of two additional patients with congenital hemolytic jaundice failed to agglutinate with the test serum. It was noted that the red blood cells from the splenic pulp of the patients with acquired hemolytic jaundice, when suspended in homologous normal serum, showed marked spontaneous agglutination but not when placed in physiologic salt solution. On the other hand, the red blood cells from the splenic pulp of two of the patients with thrombocytopenic purpura showed only rouleau formation when suspended in homologous serum. When 25 per cent human albumin was used instead of the antihuman serum rabbit serum in performing any of the positive agglutination tests already described, it was invariably found to be less sensitive or to be incapable of causing any agglutination.

#### DISCUSSION

The results of the present experiments indicate that the spleen is a source of a substance which causes the washed red blood cells of certain patients with acquired hemolytic jaundice to be agglutinated by an immune serum developed in rabbits against human serum (Coombs' serum). Presumably this agglutination is due either to the adsorption of a substance on the surface of the red blood cell or to a modification of its surface structure by contact with an active substance in the splenic pulp. On the basis of current assumptions, the agglutination test may be in fact a test for the presence of adsorbed serum globulins. Indeed, the test originally was developed for the detection of blocking antibody on sensitized red blood cells. Even so, this does not necessarily allow the conclusion that the agglutinability of the red blood cells in acquired hemolytic jaundice is the result of an immunologic process in the strict sense, that is, that the patient's red blood cells, acting as an antigen, have in some way caused the development in his own serum of specific\* antibody with avidity for the red blood cells. Although this is entirely possible, the seemingly non-specific agglutination of red blood cells, for example by antiserum developed in rabbits against Type 14 pneumococcus<sup>10</sup> or by certain viruses<sup>11</sup> with which the animal has had no previous contact, is today too well known to allow so restricted an interpretation. Indeed, perhaps the association with a variety of infections and neoplastic conditions favors the interpretation of a nonspecific adsorption of a substance by the red blood cells.

The antiserum was developed by the injection of human serum into rabbits. The property of agglutinability of the normal red blood cells, however, was probably conferred by the splenic cells rather than by the presence of serum in the splenic pulp. In the first place, the patient's serum was largely removed from the splenic pulp by the numerous washings employed in the preparations made from the fresh spleens. Second, Shen and associates<sup>12</sup> have shown that even forty-eight hour incubation with the serum from one of these patients did

\*In this paper the terms specific and nonspecific are used in order to refer to the reaction of an antibody and not to the formation of antibody. Thus an antibody formed in response to antigen A will react specifically with antigen A but may also react nonspecifically with substance B.

not cause normal red blood cells to become agglutinable by the antihuman serum rabbit serum. Third, the demonstration by Dougherty, Chase and White<sup>13</sup> that rabbit lymphocytes contain a globulin identical with normal serum gamma globulin makes it reasonable to suppose that a similar relationship obtains between cells in the human splenic pulp and the human serum used as an antigen in the preparation of the test rabbit serum.

Other evidence that the spleen is a potent source of the substance causing agglutinability of the red blood cells is the fact already cited that splenectomy in some patients with acquired hemolytic jaundice causes a decrease in the agglutinability of the circulating red blood cells. This was the case in two of these patients. Moreover, it was shown here that the washed red blood cells of the patients with acquired hemolytic jaundice when removed from the splenic pulp were always more strongly agglutinated than were those from the general circulation. On the other hand the red blood cells of two patients with congenital hemolytic jaundice, whether derived from the splenic pulp or from the peripheral blood were not agglutinated by the test serum. This negative finding in congenital hemolytic jaundice is particularly significant because there is ample evidence that in this disease the spheroidal red blood cells are selectively retained and sequestered in the splenic pulp.<sup>14, 15</sup> Consequently if a substance with affinity for the red blood cells was present in this disease ample opportunity must have existed for its action on the red blood cells in the splenic pulp to become apparent. At any rate it is clear that in these cases of acquired hemolytic jaundice in which the patients' circulating red blood cells and especially those in the splenic pulp, exhibited such a property, the spleen possessed a special capacity for causing normal red blood cells also to become agglutinable by the antihuman serum rabbit serum. In view of the short duration (two hours) of the crude exposure of the normal red blood cells to the splenic pulp *in vitro* compared with the prolonged opportunity for contact by the patients' red blood cells *in vivo* it is not surprising that the agglutination of the normal red blood cells was not more pronounced.

The red blood cells in the peripheral blood of the majority of the twelve patients with acquired hemolytic jaundice and a positive Coombs test that have been studied by us have displayed significantly increased mechanical fragility and in some instances increased osmotic fragility as well.<sup>12</sup> These findings indicate that the red blood cells have been damaged and therefore when mechanically fragile are at least unusually susceptible to destruction by the motion of the circulation. The observation that the red blood cells from the splenic pulp exhibited marked agglutination in homologous normal serum is probably the result of the presence of the adsorbed substance that under other circumstances caused the positive Coombs test. This spontaneous agglutinability of the red blood cells in the splenic pulp may be suspected as the basis of the characteristic histologic picture of congestion and infarction in the spleen that has been reported according to Dameshek and Schwartz<sup>4</sup> by several authors. It is thus possible that the passive adsorption by the red blood cells of a substance produced in the splenic pulp (and probably in other organs) may result in increasing local stagnation and injury to the red blood cells. The course of

the resulting acquired hemolytic anemia may sometimes be favorably altered by interrupting this vicious cycle by removal of the spleen. On the other hand, in other patients with a positive Coombs test splenectomy may not alter the titer or diminish the anemia, presumably because other organs share this function of the spleen.

#### SUMMARY AND CONCLUSIONS

Incubation of normal red blood cells of compatible blood group with the splenic pulp of four patients with acquired hemolytic jaundice caused these cells to become agglutinable by an undiluted immune serum developed in rabbits against human serum (Coombs' serum). When similar experiments were conducted using spleens from three patients with congenital hemolytic jaundice, three with thrombocytopenic purpura, three with congestive splenomegaly (Banti's syndrome), and two with Gaucher's disease, the results were negative.

Washed red blood cells removed from the splenic pulp of three of the patients with acquired hemolytic jaundice were more strongly agglutinated by the Coombs serum than were those derived from the peripheral blood. Similar experiments made with washed red blood cells derived from the splenic pulp or peripheral blood of two patients with congenital hemolytic jaundice resulted in no agglutination.

Red blood cells removed from the splenic pulp of three patients with acquired hemolytic jaundice exhibited marked spontaneous agglutination in homologous normal serum but not in physiologic salt solution. Similar experiments with red blood cells removed from the splenic pulp of two patients with thrombocytopenic purpura yielded negative results.

It is concluded that the spleen in certain patients with acquired hemolytic jaundice is a source of a substance responsible for the agglutination of the patient's red blood cells by antihuman serum rabbit serum. That the agglutination is probably the result of a reaction between a substance on the red blood cells and an antibody in the rabbit serum developed against human serum neither proves nor disproves that the substance on the red blood cells was elaborated by an immunologic reaction.

It is concluded that the spontaneous agglutination of the patient's red blood cells, especially when derived from the spleen, and the characteristic congestion and infarction of that organ observed by others suggest that in these instances of acquired hemolytic jaundice spontaneous agglutination of the red blood cells is a factor either causing or resulting from stagnation of these cells in the spleen. The resulting injury to the red blood cells is manifest in those that escape from their temporary sequestration in the spleen by the increased osmotic and mechanical fragilities of the circulating red blood cells of the majority of these patients. If not destroyed in the spleen, when mechanically fragile, such red blood cells may well be abnormally susceptible to the trauma of the motion of the circulation.

We are greatly indebted to Dr. Louis K. Diamond and to Dr. B. Harrison Ragle for an opportunity to carry out observations on spleens removed from their patients at operation.

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# DEMONSTRATION OF HETEROPHILE ANTIBODIES IN THE CEREBROSPINAL FLUID FROM PATIENTS WITH -INFECTIOUS MONONUCLEOSIS

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PAUL and Bunnell<sup>1</sup> in 1932 demonstrated a consistent and marked increase of heterophile antibodies in the serum of patients with infectious mononucleosis. Subsequently it was shown<sup>2, 3, 4</sup> that by absorption methods these sheep hemagglutinins could be differentiated from those present in the sera of normal individuals and in serum diseases. In 1931 Epstein and Dameshek<sup>5</sup> described a patient with meningo-encephalitis in the course of infectious mononucleosis. Since then there have been many reports of nervous system involvement with this disease.<sup>6</sup>

Several unsuccessful attempts have been made to demonstrate heterophile antibodies in the cerebrospinal fluid of patients with infectious mononucleosis.<sup>7, 10</sup> It has been emphasized<sup>11, 12</sup> that there are relatively small amounts of antibodies in the cerebrospinal fluid as compared with those in circulating blood. The purpose of this investigation was to demonstrate heterophile antibodies in the cerebrospinal fluid of patients with infectious mononucleosis by employing large volumes of spinal fluid and dilute sheep erythrocyte suspensions as antigen.

## MATERIALS AND METHODS

A 1 per cent washed sheep erythrocyte suspension was prepared and 0.1 ml of it was added to 1 ml and 0.5 ml of fresh spinal fluid in Kahn sized test tubes and shaken. Saline controls were prepared. The tubes were centrifuged for five minutes at 2,500 revolutions per minute and macroscopic agglutination was demonstrated upon resuspending the cells by shaking. Results were recorded from negative to 4 plus, depending upon the degree of agglutination.

A modification of Davidsohn's<sup>13</sup> absorption method was adopted to study the heterophilic nature of the agglutinins found in cerebrospinal fluid. One milliliter of spinal fluid was placed in Kahn sized test tubes. To one was added 0.5 ml of guinea pig kidney antigen and to the other 0.5 ml of boiled beef erythrocytes. After incubation for ten minutes at 37° C these were centrifuged at 2,500 revolutions per minute for ten minutes. One milliliter of the supernatant fluid of each was then treated in the manner described previously.

## EXPERIMENTAL RESULTS

The blood and spinal fluid of six patients with infectious mononucleosis were examined for heterophile antibodies (Table I). Lumbar punctures were done the same day or the day following the demonstration of a blood heterophile

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TABLE I A COMPARISON OF THE BLOOD AND CEREBROSPINAL FLUID HETEROPHILE ANTIBODY TITERS

PATIENT	DIAGNOSIS	BLOOD			CEREBROSPINAL FLUID				CELL COUNT	TOTAL PROTEIN (MG / 100 ML)
		HETEROPHILE ANTIBODY TITER (DAVIDSOHN METHOD)			HETEROPHILE ANTIBODY TITER (CENTRIFUGE METHOD)					
		UNABSORBED	ABSORBED		UNABSORBED		ABSORBED			
			G I K	B B E	1 ML	0.5 ML	G P K	B B E		
A	Infectious mononucleosis	1 3 584	1 1 96	Neg	2+	±	1+	Neg	4	16
B	Infectious mononucleosis	1 112	1 56	Neg	1+	Neg	Qns	Qns	15*	77
C	Infectious mononucleosis	1 448	1 224	Neg	4+	3+	2+	Neg	20†	13
D	Infectious mononucleosis	1 1 92	1 896	Neg	3+	2+	±	Neg	4	34
E	Infectious mononucleosis	1 896	1 448	Neg	2+	Qns	1+	Neg	4	42
F	Infectious mononucleosis	1 3 584	1 1 796	Neg	4+	2+	3+	Neg	2	15
G	Tuberculous meningitis	Neg			±	Neg	Neg	Neg	108	536
H	Subarachnoid hemorrhage	1 56	Neg	1 28	2+	Neg	Neg	2+	90	110
I	Trichinosis	1 112	1 56	Neg	3+	2+	2+	Neg	3	40
	Neuroblastoma									

Neutrophils 6 per cent, lymphocytes 94 per cent.

†Lymphocytes 100 per cent.

G P K, guinea pig kidney antigen

B B E, boiled beef erythrocytes

Qns, quantity not sufficient

antibody titer. The serum titers ranged from 1 112 through 1 3 584 with Davidsohn's method and the specificity was verified by his absorption procedure. A parallel series using similar dilutions of serum and the method employed in testing spinal fluids resulted in approximately an eightfold increase in these titers as compared with the usual technique of serum titration. Hemagglutinins from 1 plus to 4 plus were present in the spinal fluids of all these patients and they had the same absorption pattern as those found in the blood. The sera and spinal fluids of these patients were negative for Kahn and Kolmer Wassermann tests. The spinal fluid cell counts were increased in Patients B and C and lymphocytes predominated. No growth was obtained upon culture. The colloidal gold reactions were normal with the exception of Patient B in whom a slight mid zone curve resulted, and this may have been due to the elevated total protein.

In this study 654 routine spinal fluids were examined and nine had demonstrable sheep hemagglutinins. Six of these were from the patients with infectious mononucleosis described previously. The remaining three (0.4 per cent) so-called false positive hemagglutinins included a case of tuberculous meningitis (cerebrospinal fluid elevated protein), one of subarachnoid hemorrhage

(passive transfer of agglutinins), and one of trichinosis. Although the heterophile antibodies in the first two were absorbed with guinea pig kidney extract, the hemagglutinins in the third persisted after this absorption. The heterophile agglutinins in this patient with trichinosis remain unexplained, although the possibility of concomitant infectious mononucleosis exists.

#### DISCUSSION

A linear relationship does not appear to exist between the blood and cerebrospinal fluid heterophile antibody titers in infectious mononucleosis during the acute phase of illness. However in both the cerebrospinal fluid and blood, the sheep cell agglutinins persisted following guinea pig kidney antigen absorption and were absorbed with boiled beef erythrocytes (Table I). In a preliminary series of experiments the same spinal fluids were tested with human red cells sensitized with the virus of Newcastle disease,<sup>14</sup> and a high degree of nonspecificity was found. Encephalitis associated with infectious mononucleosis as suggested by Tidy<sup>15</sup> was noted only in Patient D whose cerebrospinal fluid hemagglutinins were not significantly higher than the others.

#### SUMMARY

A simple method is described for the demonstration of heterophile agglutinins in cerebrospinal fluid. Of 654 spinal fluids tested, six from patients with infectious mononucleosis had heterophile antibodies in both blood and cerebrospinal fluid and one of these showed nervous system involvement.

The authors wish to express their appreciation to Dr Morris L. Rakieten for advice and for conducting the experiments with the virus of Newcastle disease.

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# THE USE OF YEAST PHASE ANTIGENS IN A COMPLEMENT FIXATION TEST FOR HISTOPLASMOSIS

## II RESULTS WITH GROUND ANTIGENS

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A COMPLEMENT fixation test for the detection of antibodies against *Histoplasma capsulatum* employing the yeast phase of the organism as antigen has been described previously from this laboratory.<sup>1</sup> In the presence of yeast phase antigens, immune rabbit sera fixed complement in high dilutions, but immunologic cross reactions with *Blastomyces dermatitidis* described by others<sup>2,4</sup> working with mycelial antigens were noted also in our studies.

Yeast phase antigens described in the earlier report were completely satisfactory for short periods but tended to develop anticomplementary activity after storage for several weeks at 3 to 6° C. Also since in our complement fixation test the per cent hemolysis was determined by comparison with centrifugalized standards, it was occasionally difficult in the range nearing complete hemolysis, to differentiate nonhemolyzed sensitized sheep's cells from the residual sediment of turbid antigens. These minor difficulties instigated the search for an antigen which would meet more adequately the precise end points and demands of the test. This report describes the use of ground yeast phase antigens.

### MATERIALS AND METHODS

**Antigens**—Antigens were prepared from the yeast phase of each of three strains of *H. capsulatum* (G 2 G 5 G 6) and two strains of *B. dermatitidis* (A 1 and A 5†). The yeast phase organisms were obtained from cultures grown at 37° C on glucose cystine agar containing 35 units of penicillin and 40 units of streptomycin per milliliter of medium. After the fifth day of incubation the growth was washed gently with sterile buffered saline from the surface of at least five slants filtered through sterile gauze and centrifugalized. The sediment was resuspended in 50 ml of sterile buffered saline containing Merthiolate (1:10,000 final concentration) and stored at 37° C. Organisms thus treated were usually not viable after seventy-two hours but since further incubation did not alter the resultant antigen exposure to Merthiolate was continued as a precautionary measure for one week.

The Merthiolate killed suspension was centrifugalized for thirty minutes at 3,000 revolutions per minute, the supernate discarded and the sediment transferred to a small TenBroeck glass tissue grinder. The organisms were then ground by hand for thirty minutes. One to three milliliters of sterile buffered saline were added at five minute intervals during grinding. Finally the ground antigen mixture was centrifugalized until the supernate was completely free of organisms. This clear saline supernate constituted the antigen. The grinding procedure usually was repeated several times with each sediment and the supernates were saved.

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The G 2 G 5 and G 6 strains of *H. capsulatum* were reverted from the mycelial phase of strains 715, 95<sup>9</sup> and 650 respectively from the collection of Dr V F Conant at Duke University, Durham, N C.

† A 1 strain of *B. dermatitidis* and A 5 (Duke 930) are isolates from human cases received from Dr Conant at Duke University.

in separate containers. Supernates showing adequate and comparable titers were pooled, while those of reduced or unsatisfactory potency were discarded. After the addition of Merthiolate 1:10,000 as preservative, the pooled material was titrated and used as the stock antigen.

Antisera, diluent, sheep red blood cells, hemolysin, and complement were prepared exactly as described in the previous report<sup>1</sup> and the complement fixation test of Kent and Reim<sup>2</sup> was again employed.

### RESULTS

*Experiment 1*—The optimal dilution of each lot of antigen was determined by titration. The antigenic unit was established as being the greatest concentration contained in 0.2 ml. beyond which further increase failed to enhance the serum reaction. It was required that this antigen concentration be neither anticomplementary nor hemolytic. The optimal dilutions of the ground antigen

TABLE I. DETERMINATION OF THE OPTIMAL DILUTION OF GROUND ANTIGEN USING THE G<sup>2</sup> STRAIN OF *H. CAPSULATUM* AND SERUM PREPARED IN RABBITS AGAINST G<sup>2</sup>

DILUTION OF ANTIGEN (0.2 ML.)	POSITIVE SERUM DILUTIONS IN (0.2 ML.)						CONTROLS		
	1:10	1:20	1:40	1:80	1:160	1:320	ANTIGEN	COMPLEMENT	CELLS
1:1	0%	0%	0%	0%	0%	0%	90%	100%	0%
1:13	0	0	0	0	10	50	AC		
1:2*	0	0	0	0	20	100	100		
1:26	0	0	0	0	30	100	100		
1:4	0	0	0	30	80	160	100		
1:53	0	0	20	60	95	190	100		
1:8	5	20	50	95	100	100	100		

0 No hemolysis 100 complete hemolysis AC almost complete hemolysis

\*Optimal dilution was 1:2 since there was good fixation but no trace of anticomplementary activity.

varied between 1:2 to 1:4, and Table I shows a typical titration in which the G-2 antigen was shown to have an optimal dilution of 1:2. As can be seen in Table II, the ground antigens prepared by the described method have been used in this laboratory for twelve consecutive weeks with no development of anticomplementary activity and with no appreciable loss in titer when stored in the refrigerator.

TABLE II. DETERMINATION OF THE STABILITY OF G<sup>5</sup> GROUND ANTIGEN WHEN USED AT OPTIMAL DILUTION OVER A TWELVE WEEK STORAGE PERIOD AT 3 TO 6° C

STORAGE AT 3-6° C (WK.)	G <sup>5</sup> SERUM DILUTIONS						CONTROLS		
	1:10	1:20	1:40	1:80	1:160		ANTIGEN	COMPLEMENT	CELLS
1	0%	0%	0%	0%	20%	100%	100%	100%	0%
2	0	0	0	0	15	100	100	100	0
3	0	0	0	0	15	100	100	100	0
6	0	0	0	0	40	100	100	100	0
12	0	0	0	0	20	100	100	100	0

0 Hemolysis 100 complete hemolysis

*Experiment 2*—The results obtained with yeast phase ground antigens are demonstrated in Tables III and IV. Fixation of complement was obtained in similar titers with homologous and heterologous strains of histoplasma antigens.

TABLE III REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF GROUND ANTIGEN FROM THE G 2 STRAIN OF *H. CAPSULATUM* AND THE SERA OF RABBITS INOCULATED WITH THE G 2, G 5, AND G 6 STRAINS OF *H. CAPSULATUM* THE A 1 AND A 5 STRAINS OF *B. DERMATITIDIS* AND *B. BRASILIENSIS* S. SCHENCKII C. ALBICANS AND NORMAL CONTROL RABBIT SERUM

SERA	SERUM DILUTIONS							CONTROLS		
	1 5	1 10	1 20	1 40	1 80	1 160	1 320	ANTI GEN	COM PLE MENT	CELLS
G 2	0%	0%	0%	0%	0%	15%	50%	100%	100%	0%
G 5	0	0	0	0	5	60	100			
G 6	0	0	0	5	50	95	100			
A 1	0	0	20	40	95	AC	100			
A 5	0	15	50	90	100	100	100			
B brasiliensis	100	100	100	100	100	100	100			
S schenckii	95	100	100	100	100	100	100			
C albicans	100	100	100	100	100	100	100			
Normal	100	100	100	100	100	100	100			

0 No hemolysis (complete fixation) 100 complete hemolysis (no fixation) AC almost complete hemolysis.

The titer was taken as being the highest dilution of serum showing 50 per cent or less of hemolysis.

and antisera. For example, when the G 2 strain of *H. capsulatum* was used as the antigen source (Table III) the G 2, G 5 and G 6 rabbit antisera gave titers of 1 320, 1 80, and 1 80 respectively. With the G 5 antigen (Table IV) the G 2, G 5, and G 6 antisera yielded titers of 1 320, 1 160 and 1 160 respectively.

Sera prepared against *Candida albicans* (E 11\*), *Sporotrichum schenckii* (F 20\*), and *Blastomyces brasiliensis* (B 3†) as well as normal rabbit sera did not fix complement in the presence of histoplasma antigens. On the other hand,

TABLE IV REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING AN OPTIMAL DILUTION OF THE G 5 STRAIN OF *H. CAPSULATUM* AS ANTIGEN AND THE SAME SERA USED IN TABLE III

SERA	SERUM DILUTIONS							CONTROLS		
	1 5	1 10	1 20	1 40	1 80	1 160	1 320	ANTI GEN	COM PLE MENT	CELLS
G 2	0%	0%	0%	0%	0%	25%	50%	100%	100%	0%
G 5	0	0	0	0	0	15	70			
G 6	0	0	0	0	10	50	100			
A 1	0	0	20	40	95	100	100			
A 5	0	0	0	10	80	100	100			
B brasiliensis	100	100	100	100	100	100	100			
S schenckii	100	100	100	100	100	100	100			
C albicans	100	100	100	100	100	100	100			
Normal	100	100	100	100	100	100	100			

See footnotes to Table III

the A 1 and A 5 *B. dermatitidis* antisera fixed complement in dilutions ranging between 1 20 to 1 40 (Tables III and IV). This cross reaction in lower dilutions between the histoplasma antigen and blastomyces antisera was likewise noted and discussed in our previous report.<sup>1</sup>

**Experiment 3**—In order to evaluate further the results obtained in Experiment 2 wherein both histoplasma and blastomyces antisera fixed complement in the presence of the histoplasma antigen, ground antigens of the A 5 strain

<sup>1</sup>Isolated from patient material at the Army Medical Center

<sup>†</sup>Strain from human material received from Dr. Conant.

TABLE V CROSS REACTIONS IN THE COMPLEMENT FIXATION TEST EMPLOYING THE A 5 STRAIN OF *B. dermatitidis* AS ANTIGEN AND SERA OF RABBITS INOCULATED WITH THE A 1 AND A 5 BLASTOMYCES ANTIGENS AND THE G 2, G 5, AND G 6 HISTOPLASMA ANTIGENS

SERA	SERUM DILUTIONS							CONTROLS		
	1 5	1 10	1 20	1 40	1 80	1 160	1 320	ANTI GEN	COM PLE MENT	CELLS
A 1	0%	0%	0%	30%	100%	100%	100%	100%	100%	0%
A 5	0	0	0	10	80	AC	100			
G 2	0	0	0	0	50	95	100			
G 5	0	0	0	0	3	20	100			
G 6	0	0	0	30	65	AC	100			
B brasiliensis	95	100	100	100	100	100	100			
S schenckii	95	100	100	100	100	100	100			
C albicans	AC	100	100	100	100	100	100			
Normal	100	100	100	100	100	100	100			

See footnotes to Table III

of *B. dermatitidis* were employed against the same antisera. In the presence of the A-5 antigen both the A-1 and A-5 rabbit antisera fixed complement in dilutions of 1 40 (Table V). The G-2, G-5, and G-6 histoplasma antisera fixed complement in dilutions of 1 80, 1 160, and 1 40 respectively. With the exception of the G-5 antisera, the titers of the histoplasma antisera were lower in the presence of the blastomyces antigen than in the presence of their specific antigens (see Tables III and IV). The significance of these results will be discussed below.

#### DISCUSSION

The preparation of ground yeast phase antigens of *H. capsulatum* and *B. dermatitidis* for use in the complement fixation test is herein described. Rabbit antisera prepared from homologous and heterologous strains of *H. capsulatum* and *B. dermatitidis* fixed complement in the presence of both histoplasma and blastomyces antigens, while sera prepared against *C. albicans*, *S. schenckii*, and *B. brasiliensis* as well as normal rabbit sera did not.

The cross reaction pattern noted when the whole yeast phase organisms of *H. capsulatum* and *B. dermatitidis* were used<sup>1</sup> was even more pronounced with the use of ground yeast phase antigens. The A-1 and A-5 blastomyces antisera having specific titers of 1 40 fixed complement in the presence of ground histoplasma antigens in serum dilutions of 1 20 to 1 40. (With whole organisms this cross had been found only in dilutions of 1 10 or less<sup>1</sup>). Evidence that a strong antigenic relationship exists between these two organisms was even more apparent when histoplasma antisera was tested for complement fixing antibodies in the presence of blastomyces ground antigens. Histoplasma sera having specific titers of 1 160 to 1 320 reacted with blastomyces antigens in serum dilutions ranging from 1 40 to 1 160. However, the antiserum against the G 5 strain of *H. capsulatum* fixed complement to the same dilution in the presence of both the specific and *B. dermatitidis* antigens, while the G 2 antiserum with a specific titer of 1 320 fixed complement in the presence of blastomyces antigen only to a dilution of 1 80. This suggests that the common antigenic component of the two organisms may appear in varying degrees in different strains and emphasizes the risk involved in evaluating the results of complement fixation

tests for histoplasmosis which utilize only the antigen of *H capsulatum*. On the other hand, the use of both *H capsulatum* and *B dermatitidis* antigens allows for a more comprehensive and critical analysis of the test proper and of the results obtained therein.

The ultimate value of the complement fixation test as a potential diagnostic aid in the diagnosis of histoplasmosis in patient material is yet to be determined. Whereas the tests now in use employ histoplasmin, a filtrate of the mycelial form of *H capsulatum*, as antigen, it is our belief at this time that tests aimed toward the detection of antibodies against *H capsulatum* as it appears in pathologic processes that is the yeast phase should be investigated more fully. Immunologic cross reactions occur between blastomycetes and histoplasma rabbit antisera regardless of the phase employed as antigen but in our experience the use of histoplasmin as antigen has not been as satisfactory as yeast phase antigens because of its high incidence of anticomplementary activity and the low serum titers obtained.<sup>7</sup>

The use of whole yeast phase organisms as antigens is relatively simple and has been described earlier by us.<sup>1</sup> Ground antigens offer two advantages: (1) a clearer, more readily readable test and (2) greater stability of the antigen. A disadvantage (if it may be called such) of the ground antigen is the greater degree of cross reaction between *H capsulatum* and *B dermatitidis*. This, however, as mentioned, can usually be better evaluated by the judicious use of antigens prepared from both organisms.

#### SUMMARY

The preparation of ground antigens from the yeast phase of *H capsulatum* and *B dermatitidis* for use in complement fixation is described.

Ground yeast phase antigens are as specific as antigens made from whole organisms; they give more clear cut end points and do not develop anticomplementary activity during storage at 3 to 6° C.

The immunologic relationship between *H capsulatum* and *B dermatitidis* noted in a previous report concerning yeast phase antigens has been confirmed further.

The use of both *H capsulatum* and *B dermatitidis* antigens in evaluating complement fixing antibody responses to *H capsulatum* is recommended.

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# ANTIGENIC DIFFERENCES AMONG INFLUENZA A VIRUSES, INCLUDING SEROLOGIC RESPONSE OF PATIENTS

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THE failure of influenza virus vaccines to protect immunized individuals during the influenza outbreak that occurred early in 1947 and the demonstration of antigenic differences between the strains of virus contained in the vaccines and those responsible for the epidemic<sup>1,3</sup> have aroused further interest in the problem of strain variations among influenza viruses.<sup>1,2</sup> Strains of influenza virus were also isolated during the same epidemic when it occurred in Boston, and they too showed sharp differences from the classic PR8 strain of influenza A. The present report presents the results of studies on (1) the antigenic relationships of these recently isolated strains to several that were obtained in a local outbreak in 1943-1944<sup>6</sup> and to several other well-known strains of influenza viruses and (2) the immunologic response of patients to their homologous virus and to other strains of influenza virus.

## MATERIALS AND METHODS

*Throat Washings*—Garglings were obtained on the first to the third day of the disease from thirteen patients who were acutely ill with clinical influenza and had temperatures of 100° F or higher at the time. The washings were made with infusion broth containing 10 per cent horse serum and were collected between March 18 and April 17, 1947. Most of them were tested for virus immediately and then stored in sealed glass ampules in the dry ice cabinet. Garglings were also obtained from the patient P. W., an isolated instance of clinical influenza, on Dec. 6, 1947, the data on this patient are included in the tables, but will be discussed separately.

*Virus Isolation*—Attempts were made to isolate influenza viruses from the throat washings, and in two instances from nasal secretions, by inoculation of embryonated eggs by both the amniotic and allantoic routes as previously described.<sup>7</sup> Once the virus was established, subsequent passages all were made in eggs by the allantoic route.

*Serologic Tests*—The methods used in the hemagglutination inhibition and complement fixation tests were similar to those used in previous studies.<sup>6</sup> Serum neutralization tests were carried out in chick embryos by two different methods: one of these employed the allantoic route as described by Hirst,<sup>8</sup> and the other was a modification in which 0.25 ml. amounts of the serum-virus mixtures were injected into the yolk sac of 7-day old eggs which were then incubated for five days at 35° C, the deaths were recorded and the serum protection titers based on the LD<sub>50</sub> were calculated.<sup>9</sup> The latter method eliminated the necessity of opening each egg individually to test for the presence of influenza virus.

Acute (third day or earlier) and convalescent (tenth day or later) phase serum specimens were obtained from the patients and stored at -20° C in rubber stoppered tubes. Rabbit antisera for the study of antigenic differences were prepared against each virus. Albino rabbits, weighing 2.5 to 3.5 kilograms, were first bled to obtain control serum specimens, they were then injected intravenously with 0.1 ml. of virus-infected allantoic fluid and bled again after ten to fourteen days. The sera were heated at 60° C for twenty

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minutes and tested for antibody by the hemagglutination inhibition method. If a satisfactory rise in titer was obtained the rabbits were bled by cardiac puncture and the serums stored in sealed glass tubes at  $-20^{\circ}\text{C}$  until used.

### RESULTS

*Virus Isolations*—Strains of influenza virus were obtained from the throat washings of three patients (E B, G P and D W) of the thirteen from whom washings were obtained during the March-April 1947, outbreak and a strain also was obtained from the nasal secretions of one of them (E B). In each instance the virus was first identified on the second amniotic passage in embryonated eggs and was detected in both amniotic and allantoic fluid at that time\*. The throat washings of E B also yielded the virus after four allantoic passages. Each of the remaining throat washings and the other sample of nasal secretions were passed blindly four or five times both by the amniotic and by the allantoic routes without yielding any detectable virus. Two throat washings which had given negative results following passages in embryonated eggs were inoculated intranasally in mice and lung suspensions were passed, three times in one instance and six times in the other, without producing visible lesions.

Thus, influenza viruses were isolated only with difficulty during this outbreak, and the amniotic route of inoculation gave the best results. Similar difficulty in establishing viruses in eggs was also reported by others<sup>1</sup>.

*Serologic Tests in Patients' Serums*—The results of the hemagglutination inhibition and complement fixation tests on the serums of twenty patients who were acutely ill with clinical influenza between the middle of March and the middle of April 1947, and in the case of P W who was ill in December of that year are shown in Table I. It is seen first of all that no significant rises in titer were obtained with the Lee strain of influenza B in any instance. With the other viruses used, both the absolute titers and the extent of the rises in titer varied in the same patient with the different viruses and in a number of instances there were similar discrepancies in the titers obtained with the same virus by the two tests. Some of the latter discrepancies were elicited even when the two tests were done simultaneously with the same serum dilutions and the same virus antigens.

A fourfold or greater rise in titer was obtained by one or both tests with the PR8 strain or with one of the epidemic strains in each of the twenty patients who were ill in the spring. In five of these patients such rises were elicited either by only one of the virus antigens or by only one of the tests, but not with the other test employing the same antigen nor by either test with any of the other virus strains. The serum of one of these patients D W showed a significant rise in titer only by the agglutination inhibition test with one of the epidemic strains GP but showed either no change or only a twofold increase in titer with the other viruses used including the strain isolated from the patient's own throat washings.

The tests with the PR8 strain yielded the greatest proportion of positive results. Only one patient J M, showed no increase at all by the agglutination

\* For subsequent serologic tests with rabbit antisera and with patients' serums the viruses were used only after they had been passed through eighteen to twenty additional allantoic passages.

TABLE I RESULTS OF SEROLOGIC TESTS IN PATIENTS

PATIENT	VIRUS ISOLATION**	VIRUS ANTIGEN											
		IR 8			SWINE			EB			GP		
		H I	C F		H I	C F		H I	C F		H I	C F	
E B	+	8/64†	32/512		<4/8	16/128		32/128	—/512		<4/16	—/256	
G P	+	4/16	<4/64		4/16	8/32		32/64	4/64		8/32	<4/64	
D W	+	4/8	32/64		8/8	32/64		32/32	64/128		<4/16	32/64	
M C	0	8/32	8/32		<4/4	8/16		64/64	16/64		16/16	16/32	
M F	0	4/32	8/64		8/64	16/64		128/128	16/64		8/16	8/64	
A A	0	64/512	16/128		512/2048	32/64		64/256	16/64		4/16	16/128	
C H	0	64/128	8/32		64/256	8/32		128/128	16/64		8/32	16/32	
M P	0	128/256	32/64		128/256	16/32		128/512	8/64		16/64	32/64	
J M	0	32/32	—		64/64	—		64/32	8/32		64/32	64/256	
O B	0	8/64	32/256		16/16	AC		128/4†	AC		8/4†	AC	
D N	0	32/1024	—		—	—		64/128	8/128		—	—	
M E	—	32/64	32/128		16/64	32/256		64/128	16/128		16/8	16/128	
J J	—	64/512	4/512		64/256	—		128/512	16/256		256/256	16/64	
S A	—	8/32	<4/64		—	—		128/64	8/64		16/64	16/64	
G R	—	16/128	—		—	—		<4/4	16/16		<4/4	4/64	
V B	—	32/128	<4/32		—	—		32/128	16/16		<4/4	8/8	
M K	—	256/4096	32/512		512/4096	—		32/128	16/64		<4/16	8/32	
J W	—	16/256	4/256		<4/64	—		16/512	32/512		<4/512	—	
H B	—	16/1024	<4/128		—	—		<4/128	—		<4/64	—	
M M	—	8/32	8/8		—	—		—	—		—	—	
P W	+	8/8	4/4		—	16/16		—	8/8		—	—	

H I Hemagglutination inhibition C F complement fixation AC anticomplementary

\*+ Virus isolated 0 attempts to isolate virus failed — not attempted

†Reciprocals of dilution end point acute/convalescent (— not done)

‡Same result three times

§Agglutinin inhibition with own virus 8/8



inhibition test with this strain, and the complement fixation test was not done with this patient's serums. In this case a fourfold rise in titer was elicited only by the complement fixation test with one of the epidemic strains EB. Two patients, D W and M P, yielded only a twofold rise in titer by both tests with the PR8 strain, the latter showed a fourfold rise with two of the epidemic strains. Three other patients yielded a fourfold rise by one test with PR8 and a twofold rise by the other test with the same strain. The serums of all of the remaining patients showed a fourfold or greater rise in titer by both tests with the PR8 strain and the increases in titer were greater with this strain than with any of the others used.

Significant rises in titer were less frequent and less striking with the DW strain than with the other two epidemic strains. With each of the epidemic strains, and particularly with the EB strain the complement fixation test yielded an appreciably higher proportion of significant rises in titer than did the agglutination inhibition tests. The rises in titer with the Swine strain were less striking than those obtained with the PR8 or with the epidemic strains.

*Antigenic Relationships Among Strains of Influenza Virus*—Because of the demonstration in a previous study<sup>7</sup> that the virus neutralization test is more sensitive than either the hemagglutination inhibition or the complement fixation test for the detection of strain differences the neutralization test with rabbit antiserum was used to study the antigenic relationships of the strains isolated from this outbreak and a number of other strains. The method of Hirst<sup>8</sup> was used. The following strains of influenza virus were compared EB, GP, and DW isolated during the outbreak of March-April 1947 in Boston, FM<sub>1</sub> isolated during the epidemic of the same season at Fort Monmouth N. J. MA, MB and CP isolated during the 1943-1944 outbreak in Boston. PW isolated from a sporadic case in Boston in December 1947 and also the well known PR8, Weiss, Swine and Lee strains. The results are shown in Table II.

TABLE II. RESULTS OF IN OVO NEUTRALIZATION TESTS WITH INFLUENZA VIRUSES AND RABBIT ANTISERA USING THE ALLANTOIC ROUTE OF INFECTION

VIRUS		IMMUNE RABBIT SERUM PREPARED AGAINST										
STRAIN	DOSE†	EB	GP	DW	FM	MA	MB	CP	PR8	WEISS	SWINE	LEE
EB	500	594‡	514	32	147	<4	<4	8	<4	<4	<4	<4
GP	500	182	590	6	128	—	—	—	<4	<4	<4	<4
DW	500	27	6	113	<16	—	—	—	<4	<4	<4	<4
FM	2 825	128	446	40	152	4	16	4	<4	<4	<4	<4
MA	5 000	<4	<4	<4	<4	8	41	<4	32	8	<4	<4
MB	158	<4	<4	10	<4	32	2048	8	206	40	<4	—
CP	283	<4	<4	<4	<4	10	64	512	>4096	32	<4	<4
PR8	250	<4	<4	<4	<4	<4	64	512	2048	<4	<4	<4
Weiss	1 580	<4	<4	11	<4	—	512	—	128	128	<4	<4
Swine	1 580	<4	<4	<4	<4	<4	<4	<4	8	<4	11	<4
Lee	50	<4	<4	<4	<4	—	—	—	<4	<4	<4	256
PW§	200	<4	—	<4	<4	<4	<4	—	<4	<4	<4	<4

— Not done.

†Strains MA, MB and CP correspond to Case Numbers 63, 4 and 67 respectively in the report of the 1943-1944 epidemic.

‡Number of 50 per cent infective doses (ID<sub>50</sub>) inoculated

§Reciprocal of 0 per cent protective titer of serum homologous titer in bold type.

¶Titer of homologous rabbit antiserum 512

None of the control rabbit serums showed any neutralization with any of the serums used, even in the lowest dilution tested, namely 1:4. In the neutralization tests with the immune rabbit serums, the three strains EB, GP, and DW showed sharp differences from the classic PR8, Weiss, and Swine strains of influenza A in that there was little or no cross neutralization. Furthermore, the DW strain was distinctly different in its reaction from the related EB and GP strains, although all three of these viruses were isolated from members of the staff of the Boston City Hospital during the same outbreak. The two latter strains appear to be very closely related to the FM<sub>1</sub> strain isolated at Fort Monmouth during the same season.

The influenza virus strains of the 1947 epidemic showed little or no relationship to the three strains isolated in the 1943-1944 outbreak in Boston. Of the three strains isolated from the latter outbreak, MA shows distinct differences from the classic PR8, Weiss, and Swine strains, MB is related to the PR8 strain, and CP is almost indistinguishable from PR8 in these neutralization tests. Furthermore, strain MA is clearly differentiated from the MB and CP strains, the two latter strains show a considerable degree of relationship to each other, but do not show complete cross reactions.

*Neutralization Tests With Patients' Serums*—In the light of the demonstration of sharp strain differentiation with rabbit antisera by the serum neutralization test, it was of interest to determine whether this more sensitive test might show greater specificity in the antibody response of the human host than did the agglutinin inhibition or complement fixation tests. Selected serums from patients studied during the 1947 outbreak of influenza were, therefore, tested for neutralizing antibody content, using the yolk sac method<sup>9</sup> for this purpose. The results are presented in Table III.

TABLE III RESULTS OF NEUTRALIZATION TESTS WITH INFLUENZA VIRUSES AND SERUMS OF PATIENTS USING THE YOLK SAC ROUTE OF INFECTION

PATIENTS' SERUMS	VIRUS†				
	EB	GP	DW	FM <sub>1</sub>	PR8
EB	<4/28*	—	<4/13	<4/87	7/13 <sub>1</sub>
GP	<4/32	<4/7	<4/8	<4/38	<4/10
DW	<4/<4	<4/<4	<4/<4	8/25	4/6
HB	<4/330	—	<4/131	<4/1024	<4/516
AA	<4/29	<4/<4	<4/64	4/128	201/720
DN	<4/4	<4/9	<4/4	<4/92	32/645
PW	<4/<4	—	<4/<4	<4/<4	<4/<4

— Not done

\*Reciprocals of the serum dilutions giving 50 per cent protection (acute/convalescent)

†Inoculum per egg in most tests was about 50 LD<sub>50</sub> (range 32-63) in five days<sup>8</sup>

One striking feature of the results obtained by this method in these patients' serums was the failure of the acute phase serums to neutralize any of the viruses to any significant extent. This is in contrast to the results obtained with the hemagglutination inhibition and complement fixation tests, but corresponds with the results of the neutralization tests in the normal rabbit serums when the allantoic method was used. There were two notable exceptions in that the acute phase serums of patients A A and D N had neutralizing titers of 1:201 and

1:32 respectively for the PR8 strain low titers (1:4 to 1:8) with this strain were found also in the acute phase serums of two additional patients, and similar low titers were obtained in two patients with the FM<sub>1</sub> strain.

Among the epidemic cases, the neutralization of the viruses by the convalescent serums in this small group of adult patients failed to show clean cut or consistent strain differences. In general however, the highest titers and the greatest rises in the neutralizing titers occurred with the PR8 and the FM<sub>1</sub> strains, and this was true irrespective of whether the titers with the epidemic strains were high or low. The serums of D W failed to neutralize any of the local epidemic strains, including the one isolated from this patient's own throat washings but they did show a slight and probably significant rise in titer with the FM<sub>1</sub> strain. One patient, A A failed to show any neutralizing antibodies for the GP strain, but showed a good antibody response to all of the other strains, in another of the epidemic cases that of patient D N only minimal rises were demonstrated with the EB and DW strains and only a slightly better response was obtained for the GP virus.

*Results in a Sporadic Case*—The throat washings obtained from patient P W on Dec 6, 1947, yielded a strain of influenza virus on several separate attempts. The virus was obtained on one occasion by amniotic inoculation and was first demonstrated in the third amniotic passage. It was also demonstrated on another occasion in the first amniotic passage of the lungs of mice that had been inoculated intranasally with the washings. Both of these strains were subsequently maintained by serial allantoic passage and they gave similar reactions in all tests. The strain obtained directly in eggs was used in the tests listed in Table II. As shown in Table II this virus was neutralized only by its homologous rabbit antiserum but not by the antisera prepared with the Lee strain of influenza B the 1947 epidemic strains the 1943-1944 strains nor with any of the standard influenza A strains. The acute and convalescent serum of P W failed to show any antibody response to any of the viruses used by the hemagglutination inhibition or complement fixation tests (Table I) or by the *in ovo* neutralization tests done by the yolk sac method (Table III).

#### DISCUSSION

The data presented indicate that the outbreak of influenza which occurred in Boston in March and April 1947 was caused by strains of influenza virus which though they resembled the PR8 strain of influenza A, were antigenically distinct from that strain and also from strains isolated in Boston during 1943-1944. Similar observations have now been recorded with respect to outbreaks of influenza which occurred during the same season in other parts of this country and in England.

In addition the strains isolated during this outbreak showed some antigenic differences among themselves as indicated by cross neutralization tests in embryonated eggs with antisera prepared in rabbits. Some differences also were noted in the antibody titers and rises in titer against these strains in the serums of patients both in the *in vitro* tests used and to some extent in the *in ovo* neutralization tests done by the yolk sac method. Similar antigenic differences

also have been demonstrated among the strains isolated during the influenza epidemic in Boston in 1943-1944. The latter strains showed somewhat greater resemblance to the PR8 strain. Distinct antigenic differences among influenza B strains isolated during a single outbreak also have been demonstrated.<sup>7</sup>

Of particular interest were the occasional patients like D W who was ill during the height of the outbreak and P W who was ill in December when no other cases of influenza virus infection were established in this area. In these patients' serums little or no antibody response was detected, either with the standard strains of influenza virus or even with the strains isolated from their own throat washings. Similar observations previously have been made in several patients ill with clinical influenza shortly after the influenza A epidemic of 1943-1944.<sup>6</sup> Strains MA and CP were obtained from such cases during the latter outbreak.

In view of the distinct antigenic differentiation between the epidemic strains isolated in the spring of 1947 and the PR8 strain, it is also of interest that during this outbreak better antibody responses were elicited in the patients against the PR8 strain than against the strains isolated during this epidemic. This was true even in the patients from whom the viruses were isolated. Similar results were obtained during the same season in England.<sup>4</sup> In the present study this lack of specificity in the serologic response of adult patients was demonstrated, both by the *in vitro* tests (hemagglutination inhibition and complement fixation) and by the *in ovo* neutralization tests done by the yolk sac method. In the latter tests this was true in spite of the fact that all of the acute phase serums gave completely negative results with the three local epidemic strains. In the rabbit antiserums, on the other hand, the neutralization test permitted much more definite antigenic differentiation among the strains and proved much more satisfactory than *in vitro* tests for that purpose.

#### SUMMARY AND CONCLUSIONS

Strains of virus isolated from patients during the epidemic of influenza which occurred in Boston in March-April, 1947, were shown to be antigenically distinct from several classic strains of influenza A and from strains of influenza virus isolated in Boston during and shortly after the 1943-1944 epidemic. The strains from both of the epidemics, however, showed some antigenic relationship to the PR8 strain of influenza A, but not to the Lee strain of influenza B.

Evidence was presented which suggested that at least two antigenically distinct strains of influenza virus were active during the 1947 epidemic, one of them closely resembled the FM<sub>1</sub> strain isolated elsewhere during the same season. Antigenic differences were also demonstrated among strains of influenza A isolated in Boston during and after the outbreak of 1943-1944.

During the 1947 outbreak, higher titers and greater rises in titer of influenza antibodies were elicited in the serums of patients with the PR8 strain than with the epidemic strains.

A strain of virus isolated from a sporadic case of clinical influenza in December, 1947, showed no antigenic relationship to any of the influenza viruses included in this study. Antibodies against this strain could not be demonstrated in the serum of the patient from whom it was isolated.

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## STUDIES ON CARDIOLIPIN ANTIGEN

### IV VARIATIONS IN SENSITIVITY OF DIFFERENT LOTS OF PURIFIED LECITHIN

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IT WAS observed early that different lots of cardiolipin antigen prepared according to the same formula gave inconstant results in the standard Kahn test. The question arose whether the variable factor was present in the cardiolipin, the purified lecithin, or in both. This article briefly summarizes our serologic results based on the use of different lots of cardiolipin and lecithin.

Table I illustrates differences in sensitivity of two different lots of cardiolipin antigen in the standard Kahn test. The antigens were prepared according to a previously employed formula, namely, 0.75 per cent purified lecithin, 0.03 per cent cardiolipin, and 0.1 per cent cholesterol<sup>1</sup>. To this antigen was added 0.2 ml. of a 10 per cent solution of gum mastic (N. F.) in absolute alcohol. The titer with 0.9 per cent NaCl solution was the same with both antigens, namely, 1 plus 11. Standard Kahn tests were performed mainly with weakly positive syphilitic serums. Two readings of results were made, the first immediately after the addition of diluent (salt solution), and the second fifteen minutes later. A summation of plus signs of the readings of the three-tube tests with each antigen shows that cardiolipin antigen Lot P gave, on first and second readings, a total of 121 and 105 plus signs respectively, while antigen Lot L gave on first and second readings a total of 179 and 161 plus signs respectively. Thus, antigen Lot L was obviously more sensitive than antigen Lot P in the simultaneous examination of twenty-five serums.

This finding raised the question whether these differences in sensitivity were due to differences in cardiolipin or in purified lecithin. Table II illustrates standard Kahn results with antigens prepared with three different lots of cardiolipin and one lot of purified lecithin (Lot IA-46). Ten weakly positive serums were employed. The antigen containing cardiolipin Lot 42 gave, on first and second readings, a total of 45 and 42 pluses respectively. The antigen with cardiolipin Lot Run 3 gave, on first and second readings, a total of 43 and 42 pluses respectively. The antigen with cardiolipin Lot 35-37 gave, on first and second readings, 44 and 41 pluses respectively. It is thus evident that the three lots of antigen prepared with different lots of cardiolipin gave, with a single lot of purified lecithin, closely parallel Kahn results with weakly positive serums.

When turning to antigens containing different lots of purified lecithin with one lot of cardiolipin, the Kahn results were found to be of a different nature,

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TABLE I ILLUSTRATING DIFFERENCES IN SENSITIVITY OF TWO DIFFERENT LOTS OF CARDIOLIPIN ANTIGEN PREPARED ACCORDING TO THE SAME FORMULA

SERUM	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING			
	ANTIGEN LOT P		ANTIGEN LOT L	
	FIRST READING	SECOND READING	FIRST READING	SECOND READING
	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3
1	- 4 4	1 4 4	4 4 4	4 4 4
2	- 4 4	- 4 4	4 4 4	4 4 4
3	- 4 4	- 4 4	3 4 4	3 4 4
4	- 4 4	- 4 4	3 4 4	3 4 4
5	- 4 4	- 2 3	3 4 4	1 4 4
6	- 4 4	- 2 2	1 4 4	- 4 4
7	- 3 4	3 4 4	3 4 4	3 4 4
8	- 3 4	- 4 4	- 4 4	- 4 4
9	- 3 3	- 2 3	1 3 4	- 2 4
10	- 3 3	- 3 3	4 4 4	- 4 4
11	- 2 4	- 2 4	- 4 4	- 4 4
12	- 2 3	- 3 4	2 4 4	- 4 4
13	- 1 4	- 4 4	2 4 4	1 4 4
14	- 1 4	- 1 4	4 4 4	- 4 4
15	- 4 4	- 4 4	4 4 4	- 3 4
16	- 3 4	- 3 4	- 3 4	- 3 4
17	- 1 2	- 1 2	2 3	- 2 3
18	- 1 1	- 1 1	2 3	- 2 2
19	- 1 1	- 1 1	- 1 1	- 1 1
20	- 1 1	- 1 1	- 1 3	- 1 2
21	- 1 1	- 1 1	3 4 4	- 1 1
22 23	4 4 4	4 4 4	4 4 4	4 4 4
24 25	- 4 4	- 4 4	- 4 4	- 4 4
Total plus signs	121	105	149	161

The first reading was made after the addition of diluent the second reading was made fifteen minutes later

TABLE II ILLUSTRATING SIMILARITY IN SENSITIVITY OF CARDIOLIPIN ANTIGEN EMPLOYING LECITHIN LOT 1A 46 WITH THREE DIFFERENT LOTS OF CARDIOLIPIN

SERUM	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING					
	CARDIOLIPIN 42		CARDIOLIPIN RUN 3		CARDIOLIPIN 35 37	
	FIRST READING	SECOND READING	FIRST READING	SECOND READING	FIRST READING	SECOND READING
	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3
1	- 1 3	- 1 2	- 1 3	- 1 1	- 1 3	1 3
2	- 2 3	- 2 2	- 1 3	- 2 3	- 2 3	- 2 3
3	- 2 2	- 2 2	- 1 1	- 1 1	- 2 2	- 2 2
4	- 1 1	- 1 1	- 1 1	- 1 1	- 1 1	- 1 1
5	- 3 4	- 2 4	- 3 4	- 3 3	- 2 4	- 2 4
6	- 1 3	- 2 2	- 2 3	- 2 3	- 2 2	- 1 1
7	- 1 1	- 1 1	- 1 1	- 1 1	- 1 1	- 1 1
8	1 4 4	1 4 4	1 4 4	1 4 4	2 4 4	1 4 4
9	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
10	- 4 4	- 4 4	- 4 4	- 4 4	- 4 4	- 4 4
Total plus signs	45	42	43	42	44	41

The first reading was made after addition of the diluent the second reading was made fifteen minutes later

as is evident from Table III An antigen containing purified lecithin Lot 1A-46 gave on first and second readings 41 and 33 pluses respectively An antigen containing lecithin Lot Run 4 gave, on first and second readings, 69 and 65 pluses respectively

TABLE III ILLUSTRATING DIFFERENCES IN SENSITIVITY OF CARDIOLIPIN (P) ANTIGEN WITH TWO DIFFERENT LOTS OF PURIFIED LECITHIN

SERUM	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING			
	LECITHIN 1A 46		LECITHIN RUN 4	
	FIRST READING*	SECOND READING	FIRST READING	SECOND READING
	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3
1	- 1 3	- - 2	- 3 4	- 3 4
2	- 1 3	- - -	- 2 4	- 2 4
3	- - 3	- - 2	- 3 3	- 2 2
4	- ± 2	- ± 2	3 4 4	3 4 4
5	- 3 4	- 2 4	- 4 4	- 4 4
6	- 1 3	- 2 2	- 3 4	- 2 3
7	- 4 4	- 4 4	3 4 4	3 4 4
8	- - -	- - -	- 1 1	- 1 1
9	2 3 4	2 3 4	3 4 4	3 4 4
10	- - -	- - -	- - -	- - -
Total plus signs	41	33	69	65

\*The first reading was made after the addition of diluent the second reading was made fifteen minutes later

TABLE IV ILLUSTRATING DIFFERENCES IN SENSITIVITY OF CARDIOLIPIN (L) ANTIGEN WITH THREE DIFFERENT LOTS OF PURIFIED LECITHIN

SERUM	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING					
	LECITHIN 1A 46		LECITHIN RUN 3		LECITHIN RUN 4	
	FIRST READING*	SECOND READING	FIRST READING	SECOND READING	FIRST READING	SECOND READING
	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3	TUBES 1 2 3
1	- 2 3	- - 3	- 4 4	- 4 4	- 3 4	- - 4
2	- 3 4	- 3 3	- 4 4	- 4 4	- 4 4	- 4 4
3	- 1 4	- 2 4	- 4 4	- 4 4	- 4 4	- 3 4
4	- 2 3	- 2 3	- 4 4	- 3 4	- 3 4	- 3 4
5	- 1 3	- - 3	- 4 4	- 4 4	- 2 4	- 1 3
6	- - -	- - -	0 0 0	0 0 0	- - 3	- - 3
7	- - -	- - -	- 2 4	- 1 4	- - 1	- - 1
8	- - -	- - -	- 1 4	- - 3	- - -	- - -
9	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
10	- - -	- - -	- - -	- - -	- - -	- - -
Total plus signs	38	35	63	55	52	46

\*The first reading was made after addition of the diluent the second reading was made fifteen minutes later

TABLE V ILLUSTRATING DIFFERENCES IN SENSITIVITY OF TWO DIFFERENT LOTS OF CARDIOLIPIN ANTIGEN PREPARED ACCORDING TO THE SAME FORMULA—WITHOUT THE ADDITION OF MASTIC

SERUM	STANDARD KAHN REACTIONS WITH CARDIOLIPIN ANTIGEN EMPLOYING	
	LECITHIN LOT 17	LECITHIN LOT 11
	TUBES 1 2 3	TUBES 1 2 3
1	± 3 4*	1 4 4
2	± 4 4	- 3 4
3	- 3 4	- 3 4
4	- - 1	- 3 4
5	- - ±	- 3 4
6	- 4 4	- 2 4
7	- 2 4	- ± 3
8	- ± ±	- - 3
9	- - -	- - -
10	- - -	- - -
Total plus signs	37	36

\*One reading of the tests was made namely immediately after the addition of diluent



It seemed worth while to serologically examine different antigens containing different lots of lecithin with still another lot of cardiolipin. Lot L instead of Lot P employed in the previous experiment. Table IV summarizes the results of this comparison. The antigen with lecithin Lot 1A 46 gave in the Kahn test 38 pluses on the first reading, and 35 pluses on the second reading. The antigen with lecithin Lot Run 3 gave 63 pluses on the first reading and 55 pluses on the second reading. The antigen with lecithin Lot Run 4 gave on first and second readings, 52 and 46 pluses respectively.

A similar experiment in which two different lots of lecithin were employed with two different lots of cardiolipin in accordance with our latest formula in which 10 per cent lecithin is employed with 0.1 per cent cardiolipin and 0.025 per cent cholesterol is illustrated in Table V. No mastic was employed in this formula. It is evident from Table V that cardiolipin antigen containing Lecithin Lot 11 gave, with ten weakly positive serums a total of 56 plus signs while cardiolipin antigen containing lecithin Lot 17 gave with the same serums a total of 37 plus signs.

It is thus clear that different lots of purified lecithin in cardiolipin antigen may cause the antigen to vary in sensitivity to the extent of 40 per cent or more. These differences can be noted best when weakly positive reacting serums are employed.

Preliminary standardization methods developed in this laboratory for cardiolipin antigen indicate that many purified lecithins will become usable by variations in the ratio of lecithin to cardiolipin within certain limits. Thus with the employment of a 10:1 ratio of lecithin:cardiolipin as a working base a reduction in lecithin, such as 9:1 tends to decrease sensitivity while an increase in lecithin, such as 11:1 tends to increase sensitivity.<sup>3</sup>

#### SUMMARY

It was observed that different cardiolipin antigens, prepared according to the same formula but consisting of different lots of cardiolipin were of uniform sensitivity in the standard Kahn test. When the cardiolipin antigens were prepared with different lots of purified lecithin considerable variations in sensitivity were noted. This finding indicates that cardiolipin antigens prepared with different lots of purified lecithin require serologic standardization before their employment in tests for syphilis.

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# STUDIES ON CARDIOLIPIN ANTIGEN

## V STANDARDIZATION OF ANTIGEN FOR THE KAHN TEST

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THIS article deals with various aspects of the standardization of cardiolipin antigen for the standard Kahn test. Preliminary studies with this antigen indicated that it behaves essentially like Kahn antigen in its titration with salt solution. These studies further indicated that in the final standardization of cardiolipin antigen for use with serum this antigen also behaves essentially like Kahn antigen. Hence, many technical steps developed in connection with the titration and standardization of Kahn antigen were found to be applicable to the titration and standardization of cardiolipin antigen. The major aim of standardization of cardiolipin antigen is of course the same as that of the standardization of Kahn antigen, namely, to assure uniformity in serologic results with syphilitic and nonsyphilitic serums.

*The Need of Standardization of Cardiolipin Antigen*—Tissue extract antigen, such as Kahn antigen, requires three steps in its standardization: (1) The titration of the antigen with salt solution to determine the titer at which the antigen is to be mixed with salt solution in the preparation of the antigen suspension for use with serum. (2) Comparative tests with syphilitic and non-syphilitic serums, employing standard antigen as a control, to determine how closely a new antigen behaves like standard antigen in sensitivity and specificity. (3) Adjustment or correction of the new antigen if in its behavior with serums it is either oversensitive or undersensitive as compared with standard antigen. These three steps are necessary also in the standardization of cardiolipin antigen.

The question might arise why an antigen consisting of purified chemical reagents should need to be standardized. The need for standardization of cardiolipin antigen was pointed out in the preceding article,<sup>1</sup> namely, different lots of purified lecithin do not give rise to identical serologic results.

*A Workable Cardiolipin Antigen Formula for the Standard Kahn Test*—In attempting to develop a workable cardiolipin antigen formula for the standard Kahn test, various ratios of lecithin-cardiolipin were tried. A 25:1 ratio of these reagents first was reported with the use of a very small amount of mastic.<sup>2</sup> With the abandonment of this colloid, that ratio was found to be unsuitable for the standard Kahn test. A 20:1 ratio of lecithin-cardiolipin was then tried. Tabular results of cardiolipin antigens prepared with this ratio in their behavior with salt solution will be presented later. At present it is merely desired to state that a 10:1 ratio of lecithin-cardiolipin was found to be suitable as a working base, provided the concentration of these reagents is taken into consideration.<sup>3, 4</sup>

From the Serology Laboratory, University Hospital, University of Michigan.  
Aided by a grant from the Difco Laboratories, Detroit, Mich.  
Cardiolipin and purified lecithin employed in this study were kindly supplied by the Lederle Laboratories, Pearl River, N. Y., and by Dr. Mary Pangborn, New York State Department of Health, Albany, N. Y.

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The proper concentration of lipids plays an important role in the behavior of Kahn antigen with salt solution and with serums and it is understandable that it would also play an important role in the behavior of cardiolipin antigen. Indeed several years' trial of adjusting cardiolipin antigen to the standard Kahn test led to failure until proper concentration of reagents began to be employed. The relationship between lecithin cardiolipin ratios and the concentration of these reagents will be discussed below. First it is desired to present the basic cardiolipin antigen formula employed.

The use of the following percentages of cardiolipin purified lecithin and cholesterol results in a cardiolipin antigen which behaves broadly like Kahn antigen both in its titration with salt solution and in its reactions with serum.

Purified lecithin	1.0 per cent
Cardiolipin	0.1 per cent
Cholesterol	0.025 per cent

An outstanding feature of this formula is the relatively high concentrations of cardiolipin and lecithin and the very low concentration of cholesterol. It is believed that the 25 mg per cent of cholesterol in cardiolipin antigen matched against the 600 mg per cent of cholesterol in Kahn antigen might help to bring out selective reactivities of the two antigens in certain situations in syphilis.

*Determination of Cardiolipin Antigen Titer*—Those who are familiar with the Kahn technique<sup>5</sup> will recall that when 1 ml of the antigen is mixed with an appropriate amount of salt solution an antigen suspension is produced containing lipid aggregates. An important characteristic of these aggregates is that they are dispersible in salt solution and in serum; then in syphilitic serum new floccules appear, while in nonsyphilitic serum no floccules appear. Essentially cardiolipin antigen behaves the same way with salt solution. When appropriately mixed with salt solution an antigen suspension is produced in which the lipid aggregates are dispersible in salt solution and in serum. Then as in the case of the Kahn antigen suspension floccules appear in syphilitic serum and no floccules appear in nonsyphilitic serum.

The titer of Kahn antigen, by definition, is the smallest amount of salt solution added to 1 ml of antigen which will produce a lipid suspension containing dispersible aggregates. The same definition applies to cardiolipin antigen. However, after the titer has been obtained it is not necessarily true that either antigen at the titer will give correct results with serum. The sensitivity of the titrated antigens with serums must be determined by trial.

Briefly, the basic requirements of an antigen suspension at its titer for use in tests with serum are as follows:

- (1) The antigen suspension must contain a minimal amount of salt solution; an increase in the salt solution beyond the titer tends to reduce sensitivity and a decrease in the salt solution below the titer tends to increase sensitivity and nonspecificity.

- (2) The lipid aggregates of the antigen suspension must disperse in salt solution and in serum.

(3) When these aggregates are thus dispersed in serum, the negative reactions appear opalescent—not suggestive of cloudiness on the one hand or of water clarity on the other. The positive reactions will then show floccules of sufficient bulk as to be readily differentiable.

(4) If an antigen at its titer does not give results comparable to standardized Kahn antigen, special standardization methods are applied with a view toward bringing the antigen to standard sensitivity.

Table I illustrates the titration pictures of cardiolipin and Kahn antigens. The similarity in the titration picture of two antigens is evident. Also evident is the fact that cardiolipin antigen exhibits a narrow titration range with salt solution, while Kahn antigen exhibits a relatively wide titration range. As is illustrated in Table I, the Kahn antigen has a titer of 1 plus 13, while the cardiolipin antigen has a titer of 1 plus 0.9. When 1 ml of cardiolipin antigen is mixed with 0.8 ml salt solution instead of 0.9 ml, the resulting antigen suspension gives cloudy mixtures with serum and it is impossible to differentiate syphilitic from nonsyphilitic serums. If 1 ml of the antigen is mixed with 1 ml of salt solution, the resulting antigen suspension gives altogether too clear mixtures with serum and the suspension is of markedly reduced sensitivity. Briefly, cardiolipin antigen must be mixed with salt solution precisely at the titer, namely 1 plus 0.9, to obtain a usable antigen suspension in which syphilitic serums will show precipitates and nonsyphilitic serums will show the opalescence and clarity characteristic of negative reactions.

TABLE I SIMILARITY OF TITRATION PICTURES OF CARDIOLIPIN AND KAHN ANTIGENS

CARDIOLIPIN ANTIGEN SUSPENSIONS AMOUNTS OF ANTIGEN AND 0.9 PER CENT SALT SOLUTION (ML)	DISPERSIBILITY OF ANTIGEN SUSPENSION AGGREGATES, RESULTS IN 3 TUBE TEST, USING SALT SOLUTION INSTEAD OF SERUM	KAHN ANTIGEN SUSPENSIONS AMOUNTS OF ANTIGEN AND 0.9 PER CENT SALT SOLUTION (ML)
1 + 0.8	Cloudy, nondispersible aggregates	1 + 11
1 + 0.9*	Opalescent (titer), dispersible aggregates	1 + 13*
1 + 1.0	Too clear, dispersible aggregates	1 + 15
1 + 1.1	Much too clear, dispersible aggregates	1 + 17
1 + 1.2	Water clear, dispersible aggregates	1 + 19

\*Antigen titers employed in the Kahn test

Cardiolipin antigen Lot C11 L16-AA Kahn antigen Lot 107A

Kahn antigen, on the other hand, is likely to give results closely similar to those given at a titer of 1 plus 13 when the antigen suspensions are prepared by adding 1.2 ml salt solution to 1 ml of antigen or 1.4 ml of salt solution to 1 ml of antigen. Briefly, 0.1 ml of salt solution above or below the titration end point apparently does not markedly affect the physical properties of Kahn antigen suspensions.

This difference between Kahn and cardiolipin antigen is based on the probability that Kahn antigen contains nonantigenic colloids which are protective in nature, while cardiolipin antigen, because of its high purity, lacks these protective colloids. Fig 1 graphically illustrates the narrow titration range of cardiolipin antigen as compared with the relatively wide titration range of Kahn antigen.

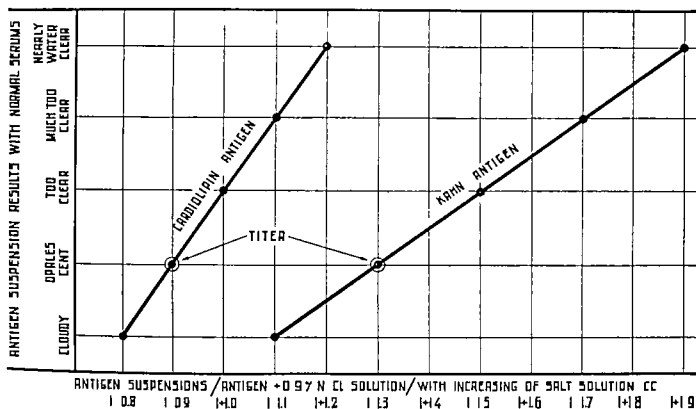


Fig 1—Graph illustrating antigen suspension of correct titer in relation to suspensions that give either too clear or too cloudy results

Table II shows that a titer of cardiolipin antigen prepared in a 10:1 ratio of lecithin:cardiolipin is obtainable only under conditions of the appropriate concentration of these reagents. When employing the same ratios of lecithin:cardiolipin, namely 10:1, but in different concentrations of these reagents, workable titers are not obtained. For example, 0.5 per cent of lecithin and 0.05 per cent of cardiolipin do not lead to a workable titer. The same holds true if 2.0 per cent lecithin and 0.2 per cent cardiolipin are employed. Evidently the 10:1 ratio of lecithin to cardiolipin in combination with the appropriate concentration of these reagents is of importance in obtaining a workable cardiolipin antigen titer for the Kahn test.

TABLE II CONCENTRATIONS OF LECITHIN AND CARDIOLIPIN (10:1 RATIO) PRODUCING AN ANTIGEN SUSPENSION USABLE IN THE STANDARD KAHN TEST

ANTIGEN PLUS SALT SOLUTION (ML.)	CARDIOLIPIN ANTIGEN FORMULA		
	A	B	C
	0.5% LECITHIN 0.05% CARDIOLIPIN 0.025% CHOLESTEROL	1.0% LECITHIN 0.1% CARDIOLIPIN 0.025% CHOLESTERYOL	2.0% LECITHIN 0.2% CARDIOLIPIN 0.05% CHOLESTERYOL
	TOTAL LIPID CONCENTRATION		
	0.545%	1.125%	2.225%
1 + 0.8	Too clear, some nondispersible aggregates	Cloudy nondispersible aggregates	Turbid nondispersible aggregates
1 + 0.9	Nearly water clear, dispersible aggregates	Opalescent* dispersible aggregates	Turbid nondispersible aggregates
1 + 1.0	Water clear, dispersible aggregates	Too clear, dispersible aggregates	Cloudy nondispersible aggregates
1 + 1.1	Water clear, dispersible aggregates	Much too clear, dispersible aggregates	Cloudy, some nondispersible aggregates
1 + 1.2	Water clear, dispersible aggregates	Nearly water clear, dispersible aggregates	Opalescent, some nondispersible aggregates

\* A usable antigen suspension was obtained with Formula B when employing 1 ml antigen plus 0.9 ml salt solution

Table III illustrates that a 20:1 ratio of lecithin-cardiolipin does not produce a workable titer at any of the three concentrations of the reagents employed, namely 0.5 per cent and 0.025 per cent, 1.0 per cent and 0.05 per cent, and 2.0 per cent and 0.1 per cent of lecithin to cardiolipin respectively

TABLE III CONCENTRATIONS OF LECITHIN AND CARDIOLIPIN (20:1 RATIO) NOT PRODUCING AN ANTIGEN SUSPENSION USABLE IN THE STANDARD KAHN TEST

ANTIGEN PLUS SALT SOLUTION (ML)	CARDIOLIPIN ANTIGEN FORMULA		
	D	E	F
	0.5% LECITHIN 0.025% CARDIOLIPIN 0.025% CHOLESTEROL	1.0% LECITHIN 0.05% CARDIOLIPIN 0.025% CHOLESTEROL	2.0% LECITHIN 0.1% CARDIOLIPIN 0.025% CHOLESTEROL
	TOTAL LIPID CONCENTRATION		
	0.55%	1.075%	2.125%
1 + 0.8	To clear, some nondispersible aggregates	Opalescent, nondispersible aggregates	Turbid, nondispersible aggregates
1 + 0.9	Nearly water clear, some nondispersible aggregates	Too clear, nondispersible aggregates	Turbid, nondispersible aggregates
1 + 1.0	Water clear, some nondispersible aggregates	Too clear, some nondispersible aggregates	Cloudy, nondispersible aggregates
1 + 1.1	Water clear, some nondispersible aggregates	Nearly water clear, some nondispersible aggregates	Opalescent, nondispersible aggregate.
1 + 1.2	Water clear, dispersible aggregates	Water clear, some nondispersible aggregates	Too clear, some nondispersible aggregates

Table IV shows the effect of increasing the cholesterol in a usable cardiolipin antigen formula on the titration results. It was found that 0.025 per cent of cholesterol did not interfere with the titration readings and was therefore adopted for use in the antigen formula. Certain lots of cardiolipin and lecithin

TABLE IV EFFECT OF INCREASING CHOLESTEROL IN USABLE CARDIOLIPIN ANTIGEN FORMULA ON TITRATION PICTURE

ANTIGEN PLUS SALT SOLUTION (ML)	AMOUNT OF CHOLESTEROL ADDED TO 1% LECITHIN AND 0.1% CARDIOLIPIN (10:1 RATIO)				
	0%	0.025%	0.05%	0.1%	0.2%
1 + 0.8	Cloudy, non dispersible aggregates	Cloudy, non dispersible aggregates	Cloudy, non dispersible aggregates	Cloudy, non dispersible aggregates	Cloudy, non dispersible aggregates
1 + 0.9	Opalescent, slightly too clear, dispersible aggregates	Opalescent*, dispersible aggregates	Opalescent, trace non dispersible aggregates	Opalescent, some non dispersible aggregates	Opalescent, some non dispersible aggregates
1 + 1.0	Too clear, dispersible aggregates	Too clear, dispersible aggregates	Too clear, dispersible aggregates	Too clear, aggregates dispersible	Too clear, some non dispersible aggregates
1 + 1.1	Water clear, dispersible aggregates	Water clear, dispersible aggregates	Water clear, dispersible aggregates	Nearly water clear, dispersible aggregates	Nearly water clear, trace nondispersible aggregate.
1 + 1.2	Water clear, dispersible aggregates	Water clear, dispersible aggregates	Water clear, dispersible aggregates	Water clear, dispersible aggregates	Water clear, dispersible aggregates

\*A usable antigen suspension was obtained when employing 1 ml antigen plus 0.9 ml salt solution

may be found usable with 0.05 per cent cholesterol, but none thus far have given satisfactory titration results with 1.0 per cent cholesterol.

*The Titer in Relation to the Sensitivity of Cardiolipin Antigen*—It was already indicated that an antigen at its titer may not necessarily give correct sensitivity results. Table V shows that cardiolipin antigens containing lecithins not approved by Dr Pangborn,\* may give titration results identical to those of antigens containing approved lots of lecithin. It is evident from Table V that lecithin Lots 12A and 13 gave satisfactory titration results similar to results of approved lots of lecithin.

TABLE V. UNIFORMITY OF TITRATION RESULTS OF SEVEN LOTS OF CARDIOLIPIN ANTIGEN EMPLOYING SEVEN DIFFERENT LOTS OF CARDIOLIPIN AND FIVE DIFFERENT LOTS OF LECITHIN, FORMULA 1 PER CENT LECITHIN, 0.1 PER CENT CARDIOLIPIN, AND 0.025 PER CENT CHOLESTEROL

CARDIOLIPIN ANTIGEN LOT	DEGREE OF DISPERSIBILITY OF LIPID AGGREGATES OF ANTIGEN SUSPENSION				
	AGGREGATES NONDISPERSI- BLE	AGGREGATES DISPERSIBLE OPALESCENT (TITER)	AGGREGATES DISPERSIBLE TOO CLEAR	AGGREGATES DISPERSIBLE MUCH TOO CLEAR	AGGREGATES DISPERSIBLE WATER CLEAR
<i>Antigen plus Salt Solution (ml)</i>					
C9 L11	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C3 L1V	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C35.3, L4	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C13 L12A*	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C6R L13	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C8 L13	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
C11 L13	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2

Lecithin not approved by Dr Pangborn.

The differences in sensitivity of cardiolipin antigens prepared with different lots of lecithin having the same titer of 1 plus 0.9 (Table V) are illustrated in Table VI. It is evident from Table VI that cardiolipin antigens prepared with lecithin Lots 12A and 13 are below the sensitivity of the other lecithin lots.

TABLE VI. COMPARATIVE SENSITIVITY OF FIVE LOTS OF CARDIOLIPIN ANTIGEN EMPLOYING FIVE DIFFERENT LOTS OF CARDIOLIPIN AND FOUR DIFFERENT LOTS OF LECITHIN FORMULA 1.0 PER CENT LECITHIN, 0.1 PER CENT CARDIOLIPIN AND 0.025 PER CENT CHOLESTEROL

SERIAL	LECITHIN					KAHN STANDARD ANTIGEN LOT 107A
	LOT 11	LOT IV	LOT 12A*	LOT 13*	LOT 13	
	CARDIOLIPIN					
	LOT 9	LOT V	LOT 13	LOT 8	LOT 11	
	ANTIGEN TITER					
	1 + 0.9 (CONTROL)	1 + 0.9	1 + 0.9	1 + 0.9	1 + 0.9	1 + 1.3 (CONTROL)
1	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4	4 4 4
2	4 4 4	4 4 4	3 4 4	3 3 4	± 3 4	4 4 4
4	3 4 4	3 4 4	3 4 4	2 2 3	- - ±	4 4 4
5	2 4 4	2 4 4	- 3 4	± 2 2	2 2 2	± 4 4
6	3 4 4	3 4 4	± 2 2	- ± ±	± ± ±	- 2 4
7	4 4 4	4 4 4	± ± 1	- ± ±	- - -	3 4 4
8	- 4 4	± 4 4	± ± ±	- ± ±	- - -	- 4 4
9	- 2 4	- 2 4	- - 2	- - 1	- 1 2	- 3 4
9	- - ±	- - ±	- - -	- - -	- - -	- - 4
10	- - ±	- - -	- - -	- - -	- - -	- - 3

\* Not approved by Dr Pangborn.

New York State Department of Health

In attempting to bring the sensitivity of different lots of cardiolipin antigen to a standard level, it was observed that this can be achieved with certain lots of lecithin by merely altering the ratio of lecithin-cardiolipin. If instead of employing a 10:1 ratio of lecithin-cardiolipin, an 11:1 ratio is employed, increased sensitivity is generally obtained. If, on the other hand, a 9:1 ratio of lecithin-cardiolipin is employed, the sensitivity is generally decreased as compared with the 10:1 ratio. Table VII illustrates the sensitivity results obtained on increasing the amount of lecithin above the 10:1 ratio and on decreasing the amount of lecithin below this ratio.

TABLE VII. EFFECT OF VARYING LECITHIN-CARDIOLIPIN RATIOS ON SENSITIVITY, FORMULAS 0.8, 0.9, 1.0, 1.1, AND 1.2 PER CENT LECITHIN (LOT 11), RESPECTIVELY, 0.1 PER CENT CARDIOLIPIN (LOT 9), AND 0.025 PER CENT CHOLESTEROL

SERUM	LECITHIN-CARDIOLIPIN RATIO				
	8:1	9:1	10:1 (CONTROL)	11:1	12:1
	ANTIGEN TITER				
	1 + 0.9	1 + 0.9	1 + 0.9	1 + 0.9	1 + 0.9
<i>Syphilitic Serums</i>					
1	444	444	444	444	444
2	344	344	344	444	444
3	111	123	344	344	444
4	±44	±44	344	344	444
5	233	234	244	244	344
6	±44	±44	144	144	344
7	---	-34	234	344	244
8	122	123	-23	±23	-34
9	---	---	-±1	-±3	-±3
10	---±	---	---	--3	--3
<i>Nonsyphilitic Serums</i>					
11	---	---	---	±---	±±±
12	---	---	---	±---	±±±
13	---	---	---	---	-±±
14	---	---	---	---	±±±
15	---	---	---	---	±±±

Occasionally it may be found that the change in the lecithin-cardiolipin ratios from 10:1 to 9:1 or 11:1 may lead to sensitivity results beyond those of standard sensitivity. In such instances one should try ratios of 9.5:1 or 10.5:1 as the case may be. Table VIII illustrates the correction of a new lot of cardiolipin antigen, undersensitive in a 10:1 ratio, by employing a 10.7:1 ratio.

It is believed that modification of the lecithin-cardiolipin ratio will not, by itself, correct all undersensitive cardiolipin antigens. It is likely that a small change in the titer, such as a slight reduction in the amount of salt solution in the preparation of the antigen suspension, will increase sensitivity. An increase in the amount of cholesterol in the antigen should also increase sensitivity. Furthermore, adjustment of the concentrations of the lecithin and cardiolipin should play a role in sensitivity. These several steps should make possible the bringing of certain undersensitive cardiolipin antigens to standard sensitivity. In view of the limited availability of cardiolipin antigens with different lecithins, it has not yet been possible to investigate all methods which may increase the sensitivity of cardiolipin antigen. It should be added that cardiolipin antigen in the standard Kahn test, in the present state of our knowledge of this antigen, is employed in this laboratory only supplementary to Kahn antigen.



TABLE VIII EFFECT OF VARYING LECITHIN CARDIOLIPIN RATIOS ON SENSITIVITY AS A METHOD IN ANTIGEN STANDARDIZATION, FORMULAS 10 AND 107 PER CENT LECITHIN (LOT 17) RESPECTIVELY, 01 PER CENT CARDIOLIPIN (LOT 11) AND 0.025 PER CENT CHOLESTERYOL

SERUM	LECITHIN		
	LOT 17	LOT 17	LOT 11
	CARDIOLIPIN		
	LOT 11	LOT 11	LOT 9
	LECITHIN CARDIOLIPIN RATIO		
	10 1	107 1	10 1 (CONTROL)
<i>Syphilitic Serums</i>			
1	$\pm 11$	$\pm 1$	$\pm 11$
2	$-\pm \pm$	$-\pm 1$	$-12$
3	$\pm 11$	$\pm 23$	$\pm 23$
4	$-\pm \pm$	$\pm 3$	$\pm 33$
5	$-34$	$\pm 34$	$\pm 24$
6	$-\pm 4$	$-24$	$-24$
7	$\pm 34$	$\pm 34$	$\pm 34$
8	$-\pm 1$	$\pm 44$	$-34$
9	$\pm 34$	$\pm 34$	$\pm 34$
10	$-24$	$\pm 34$	$\pm 34$
11	$\pm 33$	$134$	$144$
12	$444$	$444$	$444$
<i>Nonsyphilitic Serums</i>			
13 24	---	---	---

The antigen titer in each case was 1 + 0.9

#### SUMMARY

Standardization methods applicable to Kahn antigen for use in the standard Kahn test are broadly applicable also to cardiolipin antigen for use in this test. The titer of cardiolipin antigen with salt solution is obtained according to the same procedure as the Kahn antigen titer. The technique for establishing the sensitivity of cardiolipin antigen with syphilitic and nonsyphilitic serums is also similar to the technique for establishing the sensitivity of Kahn antigen. Techniques for the standardization of cardiolipin antigen to help provide uniformity in results are presented in this article.

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PROCAINE PENICILLIN, THERAPEUTIC EFFICIENCY AND A  
COMPARATIVE STUDY OF THE ABSORPTION OF SUSPENSIONS  
IN OIL AND IN OIL PLUS ALUMINUM MONOSTEARATE AND  
OF AN AQUEOUS SUSPENSION CONTAINING SODIUM  
CARBOXYMETHYLCELLULOSE

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ONE of the greatest disadvantages of penicillin is its rapid elimination from the body. In order to decrease the frequency of injections many attempts have been made to delay the absorption or excretion of penicillin and thus prolong the action of an injected dose. Until recently the most successful method of prolonging the concentrations of penicillin in the blood has been the incorporation of penicillin in peanut oil and beeswax<sup>1</sup>. With this preparation a single injection of 1 c.c. containing 300,000 units of penicillin was usually followed by assayable blood concentrations for twenty-four hours in 90 to 92 per cent of patients. The introduction of a fluid preparation obviated some of the difficulties inherent in the administration of this material<sup>2</sup>. Fluid penicillin in peanut oil and beeswax was found to be as effective as the original viscid preparation when 50 per cent of the total relative weight is made up of particles of 50  $\mu$  or more in length<sup>3</sup>. Discomfort to the patient in the form of local pain, tenderness, and nodule formation at the site of injection, however, still persisted.

For some time it has been known that a mixture of concentrated solutions of penicillin and procaine resulted in the formation of crystals which were identified as the procaine salt of penicillin. Whereas the commercially available salts (sodium, potassium, and calcium) are highly soluble in aqueous solutions and body fluids, the procaine salt is relatively insoluble. This property forms the basis of a new principle of penicillin administration. As a result of the low solubility of procaine penicillin, a repository injection of a suspension of this salt in oil or water results in delayed absorption and prolonged blood concentrations. This report presents the results of our studies on absorption following the intramuscular injection of procaine penicillin and the treatment of patients with various infections when procaine penicillin in oil was used.

#### MATERIALS

Crystalline procaine penicillin is usually prepared by the double decomposition of sodium penicillin G and procaine hydrochloride. The original commercial preparations were suspended in refined sesame or peanut oil so that 300,000 units were present in 1 c.c. as a free flowing fluid material. Such a preparation need not be refrigerated since it will remain stable for at least one year at room temperature. Because the procaine penicillin on standing separates from the oil and because of the necessity for vigorous agitation in

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order to re establish the suspension detergents such as Tween 80 and Span 80<sup>†</sup> have been added to facilitate resuspension. These measures have been only partially successful. Other investigators<sup>4</sup> have added aluminum monostearate to the mixture to maintain the suspension. The addition of aluminum monostearate to procaine penicillin in oil results in a gel formation which maintains the suspension of the penicillin in the oil.

Peanut or sesame oil was originally employed as a vehicle for injecting procaine penicillin since it was impossible to prepare injectable water suspensions of this penicillin salt. Recently it has been demonstrated that the addition of dried sodium carboxymethylcellulose to dry crystalline procaine penicillin results in a stable suspension in diluents containing water. Sodium carboxymethylcellulose in aqueous solution forms a viscous gel which maintains the procaine penicillin in discrete particulate suspension. This has eliminated the necessity for the use of oils which have been shown to be antigenic and which may cause serious complications if they are injected accidentally into a blood vessel.<sup>6</sup>

All of the procaine penicillin preparations can be withdrawn from the vial and administered through a 19 or 20 gauge needle. For the preparations containing aluminum monostearate or sodium carboxymethylcellulose dry syringes and needles are not needed. When procaine penicillin in oil is administered, moist needles and syringes may be used if the injection is made immediately after the syringe is filled. Although we have given multiple injections of procaine penicillin in oil from a single syringe without difficulty provided the withdrawal and injections were made within a very few minutes these precautions are not necessary with the preparations containing aluminum monostearate or sodium carboxymethylcellulose.

#### STUDIES ON ABSORPTION

The concentrations of penicillin in the blood at various intervals were determined according to the method of Randall and associates<sup>7</sup> following the intramuscular injection of (1) procaine penicillin in oil,<sup>‡</sup> (2) procaine penicillin in oil plus aluminum monostearate,<sup>§</sup> and (3) procaine penicillin plus sodium carboxymethylcellulose in aqueous suspension ||. The results are expressed both as percentage of patients having assayable concentrations (0.3 units per cubic centimeter or more) and as the median concentrations at the various intervals tested.

As shown in Table I all of the patients who received a single or initial injection of 300,000 units of procaine penicillin in oil (1 cc) had detectable levels at one, four, twelve, sixteen, and twenty hours. Only an occasional patient failed to have an assayable level at the twenty fourth hour. About one half of the patients had measurable levels at the thirty sixth hour and about one third at the forty eighth hour. The median levels at various hours are also shown in Table I.

While this study was in progress, several of the commercially available lots of procaine penicillin in oil were found to be inferior in that only about one half to one third of the patients had detectable levels in the blood at the twenty fourth hour following the injection of 300,000 units of procaine penicillin in oil (1 cc).<sup>8</sup> It was found that in conversion to mass production crystallization was not carefully controlled, so that large particles of procaine penicillin were

<sup>†</sup>Tween 80 Sorbitan mono oleate polyoxyalkylene derivative

<sup>‡</sup>Span 80 Sorbitan mono oleate

<sup>§</sup>Supplied by Chas. Pfizer & Company, Inc. Brooklyn, N. Y. and Eli Lilly & Company Indianapolis, Ind.

<sup>||</sup>Supplied by Bristol Laboratories, Inc. Syracuse, N. Y.

<sup>8</sup>Supplied by Wyeth Incorporated, Philadelphia, Pa.

TABLE I RESULTS FOLLOWING INTRAMUSCULAR ADMINISTRATION OF VARIOUS PREPARATIONS OF PROCAINE PENICILLIN (300,000 UNITS)

PREPARATION		HOUR											
		12	16	20	24	36	48	60	72	96	120	144	
Procaine penicillin in oil	Median levels (U/cc)	0.375	0.375	0.25	0.125	0.031	0.031						
	Percentage of patients with assayable levels*	100	100	100	96	57	36						
Procaine penicillin (particles less than 5 $\mu$ ) in oil plus aluminum monostearate	Median levels (U/cc)				0.25		0.125		0.062	0.062	0.031	0	
	Percentage of patients with assayable levels*				100		100		100	100	100	42	
Procaine penicillin plus sodium carboxymethylcellulose	Median levels (U/cc)	0.5	0.25	0.25	0.125	0.125	0.062	0.062	0				
	Percentage of patients with assayable levels*	100	100	100	100	100	100	100	25				

At least twenty-five patients were studied at each time interval indicated for the various preparations

\*Method of Randall and associates; detecting 0.03 unit per cubic centimeter and higher

produced. In grinding these particles to sizes capable of passage through 19 or 20 gauge needles, relatively large amounts of procaine penicillin dust or flour were produced. The fine particles comprising this dust or flour are dissolved and absorbed relatively rapidly so that prolonged blood concentrations are not maintained. (The manufacturers have taken steps to eliminate the fine particles from their preparations.) Therefore the blood concentrations obtained after the injection of preparations of this kind are not included in Table I.

The percentage of patients and the median blood concentrations following the injection of 300,000 units of procaine penicillin in oil plus 2 per cent W/V aluminum monostearate are also shown in Table I. It is apparent that the addition of aluminum monostearate not only stabilizes the suspension of procaine penicillin in oil but also results in prolongation of the concentrations of penicillin in the blood. Preparations containing particles of penicillin less than  $5 \mu$  in size resulted in measurable blood concentrations in all patients at twenty-four, forty-eight, seventy-two, ninety-six, and one hundred twenty hours after injection. When large particle procaine penicillin crystals are employed in this mixture the concentrations of penicillin in the blood are not so prolonged.<sup>4,9</sup>

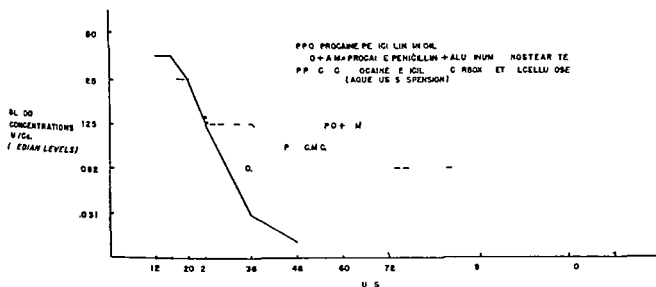


Fig 1—Curves of the concentration of penicillin in the blood following intramuscular injection of 300,000 units of procaine penicillin G suspended in various vehicles

Included in Table I are the data obtained following the injection of 300,000 units of procaine penicillin plus 3.5 Gm of sodium carboxymethylcellulose contained in 1 cc of aqueous solution. All of the patients studied had measurable levels at sixteen, twenty, twenty-four, thirty-six, forty-eight, and sixty hours. Twenty-five per cent had assayable levels at seventy-two hours.

The median concentrations at the various intervals for all three preparations have been plotted in Fig 1.

Blood concentrations were determined in seventeen patients who were receiving 300,000 units of the procaine penicillin in oil preparations every twelve hours and in fourteen subjects who received 600,000 units (2 cc) every twelve hours. Upon such regimens the blood concentrations at the twelfth hour were at least 0.125 unit per cubic centimeter and usually 0.25 and 0.5 unit per cubic centimeter. There were no significant differences between the two doses.

## CLINICAL STUDY

We have treated 251 patients with various infections with procaine penicillin in oil. All the patients received plain procaine penicillin in oil except the patients with gonorrhea who were treated with the material containing aluminum monostearate. The results and plans of therapy are summarized in Table II.

Eighty-nine patients with pneumococcal pneumonia of known type or with findings and a course characteristic of pneumococcal pneumonia were treated with 600,000 units of procaine penicillin in oil (2 c.c.) every twelve hours until they were essentially afebrile for forty-eight to seventy-two hours. The course was similar to that seen with the use of other penicillin preparations, and recovery was uneventful in all patients. These large doses were employed as a part of a study to evaluate the effect of massive doses in pneumonia. Other investigators<sup>10</sup> have found that 300,000 units a day for similar periods give satisfactory results.

TABLE II DOSAGE SCHEDULES AND RESULTS OF TREATMENT OF VARIOUS INFECTIONS WITH PROCAINE PENICILLIN IN OIL

DISEASE	NUMBER OF PATIENTS	DOSAGE SCHEDULE	COMMENT
Pneumonia	89	600,000 units b.i.d. until essentially afebrile for 48 to 72 hours	Recovered
Typed 41			
Untyped 48			
Acute bronchitis	2	300,000 to 600,000 units b.i.d. for 5 days	Recovered
Acute sinusitis	3	300,000 to 600,000 units b.i.d. for 5 days	Recovered
Tonsillitis	3	300,000 units per day for 5 days	Recovered
Scarlet fever	17	300,000 units per day for 5 days	Recovered
Vincent's infection	1	300,000 units per day for 2 days	Recovered
Infectious arthritis	3	300,000 to 600,000 units b.i.d. for 7 to 10 days	Recovered
Gonococcal 2			
Unknown 1			
Gonorrhea*	57	300,000 units	Only 1 patient had return of symptoms—possible reinfection
Syphilis	75	600,000 units per day for 5 days	All patients showed complete healing of lesions and decrease in serologic titers during 2 to 5 month follow up period
Cellulitis	1	600,000 units per day for 4 days	Recovered
Typhoid fever	1	300,000 units every 6 hours, with sulfathiazole—6 Gm. initial dose and 1 Gm. every 4 hours	No improvement

\*Patients treated with procaine penicillin in oil plus aluminum monostearate

In previous publications<sup>11, 12, 13</sup> the efficacy of penicillin in the treatment of scarlet fever has been reported. Procaine penicillin in oil in doses of 300,000 units per day for five days has resulted in prompt recovery from this streptococcal infection without pyogenic complications in all seventeen patients treated.

Three patients with bacterial arthritis were treated. Two were considered to have had gonococcal arthritis, since gonococci were isolated from a coexistent

urethral discharge One of the patients was treated with 300,000 units of procaine penicillin in oil every twelve hours Only slight improvement was noted after five days at which time the dose was increased to 600 000 units every twelve hours and rapid improvement ensued The second patient received 300 000 units every twelve hours for seven days with good results These patients are included in a recent report <sup>14</sup> The third patient was a young colored woman, six months pregnant, who had a profuse vaginal discharge and arthritis of the wrists Gonococci were not isolated from cultures of the vaginal discharge The patient was given 300,000 units of procaine penicillin in oil every twelve hours for seven days with rapid regression of the cervicitis and arthritis

Two patients with acute bacterial bronchitis and three with acute sinusitis have been treated successfully One of the patients with acute sinusitis also had acute catarrhal otitis media, the causative organism being a pneumococcus Type 4 This patient was given 600 000 units (2 c c) of procaine penicillin in oil per day for four days The temperature dropped to normal within twelve hours the nasal discharge became less purulent and viscid and was gone at the time treatment was discontinued

A patient with Vincent's infection of the mouth received 300 000 units per day for three days Relief from soreness was reported within twelve hours and the gums appeared normal at the forty eighth hour

Three patients with acute follicular tonsillitis were treated with 300,000 units of procaine penicillin in oil per day for five days Pain on swallowing and soreness subsided rapidly and the exudate promptly disappeared A beta hemolytic streptococcus was isolated from the throat of one patient before treatment was started and was not found thereafter

In the patient with typhoid fever the bacteriologic diagnosis was made on the fourth hospital day After several days during which time there was no improvement on other therapy it was decided to institute a routine similar to that used by Comerford and co workers<sup>15</sup> in the treatment of typhoid carriers Six grams of sulfathiazole were given initially followed by 1 Gm every four hours In addition 300 000 units of procaine penicillin in oil were given every six hours The strain of typhoid bacillus which was recovered from the blood cultures was found to be resistant to more than 20 units per cubic centimeter of penicillin whereas the blood penicillin concentrations prior to injection at the twelfth hour were found to be between 1 and 2 units per cubic centimeter of serum There was no improvement after five days and therapy was discontinued This patient developed an abscess on the buttock from which the typhoid organism was recovered The source of this local infection was not established although it is possible that the bacteria were carried in from the skin by the needles during injections of procaine penicillin This patient later recovered on symptomatic treatment

We have treated fifty seven patients with acute gonorrhea using a single injection of 300 000 units of procaine penicillin in oil plus aluminum monostearate The patients were followed for a period of twenty one days All the patients recovered except one who suffered a possible relapse during the third posttreatment week It is believed however that this patient was re exposed during the interim

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Another advantage of procaine penicillin preparations is the paucity of local reactions. Beeswax, which acts as a foreign substance and which may be antigenic, has been eliminated. We have not been able to demonstrate any sensitivity to procaine penicillin.

The maintenance of continued blood concentrations makes procaine penicillin in oil more desirable in the treatment of infections requiring short courses and more feasible in the cases requiring long courses. The results in the patients treated have been good. The only patients in whom no improvement followed the administration of procaine penicillin were the patient with typhoid fever and the one with acute gonorrhea who probably had a reinfection.

#### SUMMARY AND CONCLUSION

A new penicillin salt, procaine penicillin, incorporated in oil in oil and aluminum monostearate and with sodium carboxymethylcellulose in an aqueous solution has been studied.

The concentrations of penicillin in the blood at various intervals have been determined following intramuscular injections. The results are expressed both as percentage of patients having assayable levels and as the median levels at the various intervals tested.

Minimal local reactions and no systemic reactions were observed even though twenty nine patients received a second course.

Therapeutic results in patients with various infections were similar to those obtained with penicillin in other dosage forms.

It is concluded that procaine penicillin is a superior preparation for repository penicillin therapy since it does not require the use of dry syringes and needles, is followed by very few local reactions and results in more prolonged blood penicillin concentrations than any penicillin preparation yet studied.

We wish to thank Dr. Mark H. Lepper, Dr. Robert L. Brickhouse, and Dr. Thomas E. Stone for clinical assistance, and Mrs. Joan Brody, Miss Myrtle I. Meyer, Miss Helen Wright, and Miss C. Barbara O. Neil for technical assistance.

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# ACQUIRED RESISTANCE OF PSITTACOSIS VIRUS TO SULFADIAZINE AND EFFECTS OF CHEMICAL ANTAGONISTS ON SULFONAMIDE ACTIVITY

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THE susceptibility of members of the psittacosis lymphogranuloma group of viruses to sulfonamide activity has been shown to vary considerably depending on the virus strain, the drug tested and the host tissue employed<sup>1 2</sup>. Among the viruses found to be susceptible to sulfadiazine was the 6BC strain of psittacosis virus<sup>3 6</sup> originally isolated by Dr K F Meyer. It was shown that although chick embryos and mice could be protected from the lethal effect of the infection, active virus could be recovered from the tissues in most instances indicating that growth was not completely inhibited. Jones Rake and Stearns<sup>7</sup> isolated lymphogranuloma venereum virus from mice treated with different sulfonamides and retested these strains in other mice maintained on a diet containing either sulfathiazole or sulfadiazine. Four out of eight strains appeared to show an increased resistance to the drug therapy.

It was considered of interest to investigate further the susceptibility of the 6BC strain of psittacosis virus to sulfadiazine to test the effect of para aminobenzoic acid (PABA) and pteroylglutamic acid† (PGA) on sulfonamide activity, and to develop a strain with increased resistance to sulfadiazine.

## MATERIALS AND METHODS

The 6BC strain of psittacosis virus was used throughout this study. Yolk sac passages were maintained as 10 per cent suspensions by weight in nutrient broth, frozen and stored in a dry ice chest. From these stock preparations further tenfold dilutions were made in broth as desired, considering the 10 per cent suspension as a 10-1 dilution. Eight to nine day old embryonated eggs from White Leghorn chickens were used for inoculation into the yolk sac. LD<sub>50</sub> estimations of virus activity were performed by the single dilution method<sup>8</sup> in which the average day of death of a group of eggs is used as the basis for the end point employing previously determined standard curves for this strain of virus. Eggs were candled daily for ten days at twenty four hour intervals from the time of inoculation. In many instances a delay in the average day of death was the only available criterion of drug effect although if an effective drug was employed in higher concentrations differences could be expressed in terms of per cent of surviving eggs.

Sodium sulfadiazine, sulfathiazole, sulfanilamide, sulfamerazine and para aminobenzoic acid were dissolved to the desired concentration in distilled water and sterilized by filtration through fritted glass filters. Just prior to inoculation the drug solution and the virus suspension in broth pH 7.4, were mixed in the desired proportions. The pteroylglutamic acid was made up as its sodium salt in distilled water and inoculated into the eggs one half hour before the virus.

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†The pteroylglutamic acid used was a commercial preparation Folvite manufactured by Lederle Laboratories.

## RESULTS

*Susceptibility of the Normal 6BC Strain of Psittacosis Virus to Sulfadiazine (SD)*—Stock seed of 6BC yolk sac virus of known titer in eggs was diluted in broth to  $10^{-3}$  through  $10^{-6}$ . Equal volumes of these virus suspensions were mixed with solutions of sulfadiazine containing 0.5, 0.25, or 0.125 mg per 0.1 ml or with sterile distilled water as controls. Groups of twelve eggs were inoculated with 0.2 ml of these mixtures by the yolk sac route. On the tenth day of the experiment the yolk sacs were harvested from four living eggs of each group receiving 0.5 mg of sulfadiazine. To determine the virus concentration of the membranes at this period, each yolk sac pool was ground with sterile glass beads, diluted with sterile broth to a  $10^{-2}$  dilution, and inoculated into thirty eggs. The results are shown in Table I.

TABLE I. SUSCEPTIBILITY OF THE 6BC STRAIN OF PSITTACOSIS VIRUS TO VARYING CONCENTRATIONS OF SULFADIAZINE

INOCULUM		D/T*	AVERAGE DAY OF DEATH	LD <sub>50</sub> OF VIRUS FROM LIVING EGGS ON TENTH DAY
VIRUS (LD <sub>50</sub> DOSES)	SULFADIAZINE (MG)			
10 <sup>4.5</sup>	0.5	1/12	9.0	10-6.40
	0.25	11/12	8.0	-----
	0.125	11/11	5.6	-----
	None	10/10	4.2	-----
10 <sup>3.5</sup>	0.5	2/10	9.5	10-6.80
	0.25	8/9	8.1	-----
	0.125	9/9	6.9	-----
	None	11/11	5.1	-----
10 <sup>2.5</sup>	0.5	0/10	--	10-5.90
	0.25	7/11	8.3	-----
	0.125	12/12	8.2	-----
	None	12/12	5.4	-----
10 <sup>1.5</sup>	0.5	1/12	10.0	10-5.0
	0.25	4/10	9.5	-----
	0.125	5/10	9.4	-----
	None	11/11	5.9	-----

\*Dead mice per total inoculated

†Pooled yolk sac material from four eggs of groups inoculated with 0.5 mg of sulfadiazine

A decrease in the concentration of the virus inoculum resulted in a more marked effect of the drug. However, even with the most concentrated virus inoculum, 10<sup>4.5</sup> LD<sub>50</sub> doses, a definite delay in death of the embryos was evident with 0.125 mg of sulfadiazine, although the mortality was 100 per cent. The LD<sub>50</sub> figures in the last column show that although many embryos were protected by 0.5 mg of sulfadiazine, in each case active virus was recoverable from the yolk sacs in considerable amount.

*Antagonistic Action of Para-Aminobenzoic Acid (PABA) and Pteroylglutamic Acid (PGA)*—Para-aminobenzoic acid as a competitive antagonist of sulfonamides is well documented in experiments with bacteria.<sup>9</sup> Fendley<sup>10</sup> further reported an experiment in which the effect of sulfanilamide on lymphogranuloma venereum virus administered intracerebrally in mice was antagonized considerably by para-aminobenzoic acid in the diet. Subsequently, Secker

Graessle, and Dusenbery<sup>11</sup> were unable to confirm Findlay's results and suggested that the mode of action of the sulfonamides on lymphogranuloma venereum virus differed from their action on other susceptible agents.

Pteroylglutamic acid also has been shown to antagonize sulfonamides, but this compound acts probably in a noncompetitive manner and is quantitatively less effective than para aminobenzoic acid.<sup>12, 13</sup>

Shortly after the completion of our work a report by Morgan<sup>14</sup> appeared in which antagonism of sulfadiazine inhibition of psittacosis virus by para aminobenzoic acid and pteroylglutamic acid was reported. A competitive inhibition by para aminobenzoic acid was indicated whereas pteroylglutamic acid was said to be noncompetitive in action. As little as 0.05 mg of pteroylglutamic acid was reported to be sufficient to antagonize 25 mg of sulfadiazine in eggs infected with 10,000 LD<sub>50</sub> doses. Apparently survival or death of the eggs during the ten day observation period was the only criterion considered for antagonism of the sulfadiazine activity. From the tabulated results 0.1 mg of pteroylglutamic acid antagonized (all eggs dead) as much as 50 mg of sulfadiazine. Our results are similar to those of Morgan with para aminobenzoic acid but may differ somewhat in the experiments with pteroylglutamic acid as will be seen from the data presented below.

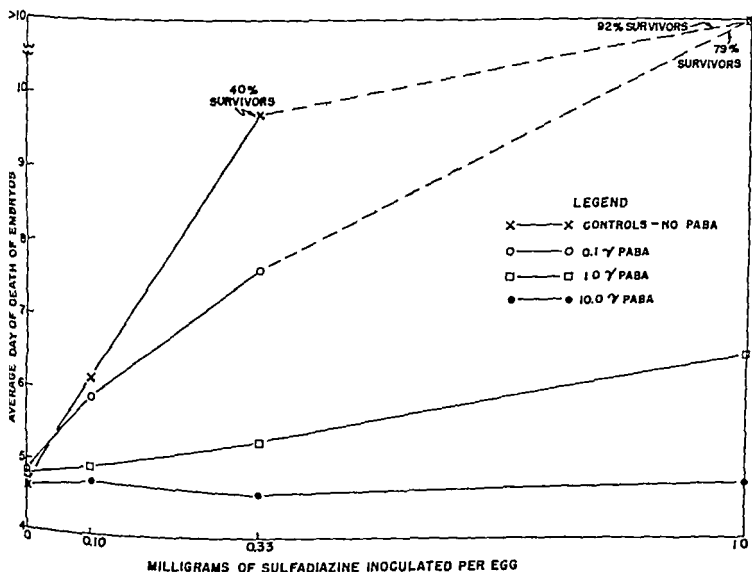


Fig. 1—Antagonism of sulfadiazine by para aminobenzoic acid with psittacosis virus in eggs

Para-aminobenzoic acid was tested quantitatively for its antagonistic activity to sulfadiazine, employing the 6BC strain of psittacosis virus in eggs. The stock virus was diluted to  $3 \times 10^{-4}$  in M/15 phosphate buffer solution at pH 7.5. To aliquots of this virus suspension were added, just before inoculation, equal volumes of sulfadiazine solution (1, 0.33, or 0.1 mg per 0.1 ml) and para-aminobenzoic acid solution (10, 1.0, or 0.1  $\gamma$  per 0.1 ml). Distilled water was substituted for the chemicals in the control tubes. Each mixture was inoculated into thirty 8-day-old eggs by the yolk sac route, using 0.3 ml per egg. The eggs were candled daily for the usual period of ten days. Fig 1 shows the results obtained in terms of the average day of death of each group of eggs.

It can be seen by comparison of the values for the average day of death at the different sulfadiazine levels that complete or almost complete antagonism occurred with 10  $\gamma$  of para-aminobenzoic acid against 1 mg of sulfadiazine and with 1  $\gamma$  of para-aminobenzoic acid against 0.1 mg of sulfadiazine, which is evidence of competitive action. Even 0.1  $\gamma$  of para-aminobenzoic acid manifested some antagonistic effect in the presence of 0.33 mg of sulfadiazine, the average day of death was reduced from 9.7 in the control eggs to 7.6 in the test eggs. The relation between the concentration of sulfadiazine and the average day of death appeared to fall in a linear fashion within the ten day observation period.

TABLE II ANTAGONISM BY PTEROYLGLUTAMIC ACID OF SULFADIAZINE ACTIVITY AGAINST PSITTACOSIS VIRUS, 10,000 LD<sub>50</sub> DOSE

PGA (MG)	SD (MG)	A D D *	MORTALITY RATIO	PER CENT SURVIVORS
None	None	4.20	25/25	0
None	1	8.00	3/20	85
None	5	--	0/22	100
0.1	None	4.03	22/22	0
0.1	1	7.00	25/26	4
0.1	5	8.00	12/25	52
0.5	None	4.12	25/25	0
0.5	1	4.55	22/22	0
0.5	5	4.43	23/23	0

\*Average day of death living eggs not included

Comparable experiments with pteroylglutamic acid as antagonist showed this compound to be less effective than para-aminobenzoic acid. Morgan's results, however, prompted investigation of higher concentrations than first tested by us. Accordingly, 0.1 and 0.5 mg of pteroylglutamic acid, in the form of its sodium salt, were tested against 1 and 5 mg of sulfadiazine in 7 day old eggs as used by Morgan. In order to duplicate conditions, the pteroylglutamic acid and sulfadiazine were inoculated as a mixture in a volume of 0.25 ml, followed by inoculation of approximately 10,000 LD<sub>50</sub>. Results are shown in Table II in which the average day of death is listed along with the per cent survivors as an indicator of antagonism of the sulfadiazine activity.

It can be seen that 0.5 mg of pteroylglutamic acid antagonized either 1 or 5 mg of sulfadiazine to the extent that all the eggs died and the average day of death (4.43 and 4.55) closely approached the figure for the control group of

that series (4.12) Since it has been shown<sup>8</sup> that the growth rate of the virus in the yolk sac is mirrored in the average time of death this measurement is considered to be a more delicate criterion for the degree of antagonism exerted. If virus growth is allowed to continue in a normal fashion the average day of death of the test eggs will be very close to that of the control group and would indicate complete antagonism. With 0.1 mg of pteroylglutamic acid although considerable antagonism is evident against 1 mg of sulfadiazine where only one of twenty six eggs survived the average day of death was 7.00 as compared with 4.03 for the control. Against 5 mg of sulfadiazine approximately half of the inoculated eggs survived and the remainder showed an average day of death of 8.00. These results indicate that the antagonism by pteroylglutamic acid is not complete unless heavy concentrations are employed. When 0.1 mg of pteroylglutamic acid was tested against 50 mg of sulfadiazine eleven out of eighteen eggs survived and the eggs that died had an average day of death of 8.30 compared with 4.23 for the controls. It is possible that free para amino benzoic acid as an impurity in the pteroylglutamic acid preparations may be a factor in the activity shown.

It should be mentioned also that in every instance in which pteroylglutamic acid was inoculated with the virus in the absence of sulfadiazine the average day of death of the eggs was slightly lower than that of the controls. Although the differences were small between 0.1 and 0.5 days they were consistent and suggest a slight stimulating effect of this compound on the growth of the virus.

Since the reports in the literature<sup>10, 11</sup> concerning the antagonism of para aminobenzoic acid to sulfonamides with lymphogranuloma venereum were contradictory, an attempt was made to test the effect of this antagonist against sulfadiazine using lymphogranuloma venereum virus in embryonated eggs. Stock lymphogranuloma venereum virus of yolk sac origin was diluted to  $10^{-2}$ . Equal volume mixtures of the virus suspension with sulfadiazine solution (0.2 mg per 0.1 ml) and/or para aminobenzoic acid solution (0.01 mg per 0.1 ml) were prepared and inoculated into groups of thirty 8 day old embryonated eggs by the yolk sac route. Distilled water was substituted in the control tubes. The eggs were candled daily for ten days.

Although all the eggs inoculated with the control preparations that is virus alone or virus with para aminobenzoic acid were killed none died of those receiving virus plus sulfadiazine and only two of twenty seven eggs died of those receiving both sulfadiazine and para aminobenzoic acid with the virus. Further duplicate yolk sac pools of living eggs collected from each group on the tenth day of incubation did not indicate any significant differences in virus content between those eggs receiving an inoculum of sulfadiazine alone and those receiving sulfadiazine with para aminobenzoic acid. Thus it is apparent that the para aminobenzoic acid did not antagonize the action of the sulfadiazine as it did when psittacosis virus was employed as the test agent. These results tend to confirm the negative findings of Seeler and co workers<sup>11</sup> with this agent with the mouse as test animal.

*Development of a Sulfadiazine Resistant Strain of Psittacosis Virus*—In order to develop a resistant strain the stock seed of 6BC virus diluted usually

to  $10^{-3}$ , was passed five times by the allantoic route in the presence of 0.5 mg of sulfadiazine. Very few of the eggs thus inoculated died during the ten day observation period, so allantoic fluid from living eggs was harvested for passage on the third to the eighth day. Little or no increase in resistance to the sulfadiazine was noted during this period upon test either by the allantoic or yolk sac route. It was again shown, however, that the allantoic fluid from living eggs inoculated with virus and sulfadiazine harbored active virus, often in considerable concentration, titrating to  $LD_{50}$  values as high as  $10^{-3}$ . Yolk sac material was harvested from five eggs, dead on the tenth day, which had received an inoculum of virus from the fifth sulfadiazine passage, plus 0.5 mg of sulfadiazine. This passage virus, 6BC-SD-6, when compared with the parent virus in the presence of 0.5 or 0.25 mg of sulfadiazine, showed some increased resistance as manifested by a reduction in the average day of death. When the daily growth of this passage virus in the yolk sac was compared with that of the parent strain in the presence and absence of 0.5 mg of sulfadiazine, the curve of the 6BC-SD-6 strain lay higher than that of the normal strain in the presence of sulfadiazine, but lower than in its absence. This indicated only partial resistance of this passage virus to the drug. This strain was then passed four more times in a  $10^{-2}$  dilution by the yolk sac route in the presence of 0.5 mg of sulfadiazine, employing yolk sac material harvested from dead eggs on the third to the fifth day. The final harvest, having been passed a total of ten times in the presence of this drug, was labelled 6BC-SD-10.

The  $LD_{50}$  titer of this virus in eggs by the yolk sac route was  $10^{-8}$ . The growth curves of this strain were compared with those of a parent stock strain of the same initial titer. Each virus was diluted to a  $10^{-3}$  dilution in broth and mixed with an equal volume of sulfadiazine solution containing 0.5 mg per 0.1 ml or distilled water as a control. Groups of 8-day-old eggs were inoculated by the yolk sac route with 0.2 ml, and at intervals of four hours and of one, three, four, and six days yolk sac pools were harvested from four live eggs of each group as long as they were available. These pools were then each titrated by the single dilution method in twenty-four eggs. The results are shown in Fig. 2.

It is apparent that the growth of the sulfadiazine-resistant strain of virus, 6BC-SD-10, in the presence of 0.5 mg of the drug was practically identical with that of the same strain or the stock strain in the absence of sulfadiazine. All of these eggs were dead by the fifth day. On the other hand, the stock strain in the presence of the sulfadiazine showed definitely slower growth and, among a group of thirty separate eggs of this series, there were 41 per cent survivors at the end of ten days. This strain was further shown to have developed complete resistance to 0.2 mg amounts of sulfathiazole and sulfamerazine. Sulfanilamide, in the same concentration, had no significant effect on the parent strain, so that no difference was detectable with this compound.

Following transfer of the 6BC-SD-10 strain through ten rapid passages by the yolk sac route in eggs in the absence of sulfadiazine, the titer in the yolk sacs reached approximately  $10^{-10}$ . When retested for its activity in the presence of 0.5 mg of sulfadiazine, it was found to have retained complete resistance to



this concentration of the drug. It was then tested against concentrations of sulfadiazine as high as 20 mg. per cc. and complete resistance was still evident. Since considerable virus of a normal strain is enabled to grow out in the presence of sulfonamides, it is possible that selective breeding plays an important role in the development of a highly resistant progeny by repeated passage.

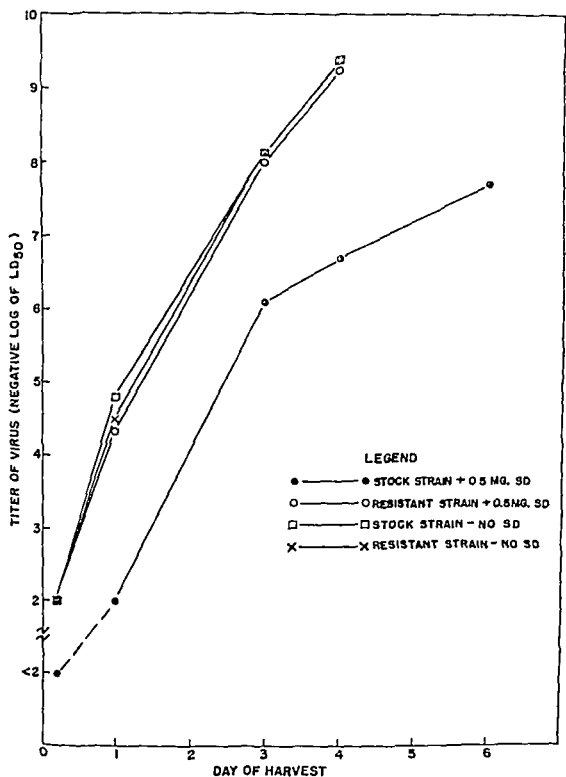


Fig. —Growth curves of stock strain and resistant strain of psittacosis virus in the presence and absence of sulfadiazine.

An allantoic fluid preparation of the resistant strain was partially purified by several cycles of centrifugation and examined under the electron microscope. No significant differences in morphology were found to distinguish it from similar preparations of the parent virus.

## SUMMARY

The 6BC strain of psittacosis virus, although quite susceptible to the action of sulfadiazine in eggs as shown by reduced mortality, multiplied to a considerable extent in the presence of the drug.

Para-aminobenzoic acid was found to be a highly effective antagonist of sulfadiazine with this virus in eggs, and a competitive relationship was suggested. A similar test with the virus of lymphogranuloma venereum did not show any antagonistic effect.

Pteroylglutamic acid was found to require considerably higher concentrations for demonstration of complete antagonism against as little as 1 mg of sulfadiazine, although partial antagonism was demonstrable against 50 mg of sulfadiazine.

By repeated passage of the 6BC virus in the presence of sulfadiazine, a strain was developed which was completely resistant to 20 mg of the drug even after ten passages through normal eggs. Concomitant resistance of this strain to sulfathiazole and sulfamerazine was also demonstrated. No morphologic differences between the resistant strain and the parent virus were observed with the electron microscope.

The author gratefully acknowledges the technical assistance of Miss Vivian Andrew and John C. Young, PhM3c.

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# THE EFFECT OF NICOTINIC ACID AMIDE ON EXPERIMENTAL TUBERCULOSIS OF WHITE MICE

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## INTRODUCTION

IN SCREENING some two to three thousand chemicals against the tubercle bacillus on the chorioallantoic membrane of the chick embryo we<sup>1</sup> found that 15 per cent of the compounds were inhibitory. Later all of these active chemicals were screened according to a technique that utilizes white mice. This technique was introduced by two Russian workers, Shpanier and Chertkova<sup>2</sup> in 1944 and by Youmans and McCarter<sup>3</sup> in this country in 1945.

In the Russian report the mice were infected by an intravenous injection of 0.5 mg of a 2 week old culture of the human type of tubercle bacillus, H<sub>37</sub>R<sub>v</sub>. They were treated with intramuscular injections of the chemical suspended in oil, receiving ten such injections in fifteen days. Evaluation of the results was made by culturing a suspension of the parenchymal organs on egg medium and after one month examining for macroscopic growth of the tubercle bacillus.

In this country Youmans and McCarter<sup>3</sup> developed the technique of producing experimental tuberculosis in mice and established a readily reproducible test. Thus they contributed a convenient *in vivo* method of screening a large number of compounds. They injected intravenously 0.1 mg of a TB suspension made from a 3 week old culture. Youmans<sup>4, 5</sup> used this technique with considerable success in demonstrating the activity of streptomycin and later the activity of *para* amino salicylic acid.

## EXPERIMENTAL

Swiss mice\* weighing approximately 17 to 20 grams were used in our experiments. These were infected intravenously with 0.25 mg of a TB suspension (H<sub>37</sub>R<sub>v</sub>) made from a 14 to 18 day old culture. Animals so infected and left untreated usually died in three to three and one half weeks. Though there was no apparent loss of weight in the first one to two weeks, the animals lost weight in the latter stage of infection, and at the time of death they usually weighed 15 to 18 grams. For the most part oral treatment of infected animals was begun on the day after inoculation. The compounds were administered in the diet. The diet consisted of ground Rockland mouse pellets.

*Nature of Infection*—Though the bacteria were given intravenously, there was no generalized military tuberculosis. Instead the disease seemed to be centered mainly in the lungs, the other organs such as the liver, spleen, and

From the Lederle Laboratories Division, American Cyanamid Company.

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Obtained from Tumblebrook Farm, Brant Lake, N. Y.

TABLE I THE VITAMIN OF ENTERICULOSIS OF WHITE MICE WITH DERIVATIVES OF NICOTINIC ACID

CHEMICAL NUMBER	CHEMICAL NAME	ILLICIT CHEMICAL IN DIET	AVRAGE BEGINNING WEIGHTS (GM)	AVRAGE WEIGHTS (3 WKS)	PERCENT SURVIVAL	GROSS AREA OF EXPOSED LUNG†	AMOUNT OF DISEASE SHOWN IN LUNG SECTION†	ACTIVITY OF PREPARATION CHEMICALLY
	None (uninfected mice)	None	19.4	22.6	100	0	0	
A-17N	Stictopycnin*	None	19.8	20.1	100	0	± 1	Active
606L	Nicotinic acid amide	0.75	18.4	21.0	90	1	1	Active
		0.5	18.4	22.6	100	1	2	Sl Active
525L	N Nicotinyl 3 aminopyridine	0.5	19.0	16.1	70	2.3	2.3	V Sl Active
526L	N (2 7 diazohyl) nicotinamide	0.12†	19.1	17.9	60	2.3	2	V Sl Active
528L	N (2 Pyrimidyl) nicotinamide	1.5	19.0	16.2	40	2.3	2.3	V Sl Active
738L	N Isopropyl nicotinamide	0.25	18.8	16.1	10	2.3	2	V Sl Active
732L	N Butyl nicotinamide	0.25	19.8	19.0	10	2.3	2.3	V Sl Active
644L	N 2 Piperidyl propyl nicotinamide	0.25	19.0	19.5	50	2	2.3	V Sl Active
665L	N Nicotinyl derivative of impure Merfand	0.25	19.5	17.0	0	3	3	V Sl Active
637L	N Nicotinyl 1 aminoanthraquinone	0.25	20.8	19.3	100	2	3	None
638L	N Cyclohexyl nicotinamide	0.25	19.3	17.6	10	4	4	None
640L	N Dodecyl nicotinamide	0.25†	19.6	17.0	20	4	4	None
642L	5 (Nicotinyl amino) 2 methyl coumarone	0.25	19.0	19.0	20	4	4	None
650L	Nicotinyl coumarone	0.25	20.1	19.0	50	4	4	None
641L	N Nicotinyl benzylamine	0.25	20.1	15.0	0	4	4	None
645L	4 (Nicotinyl amino) salicylic acid	0.25	20.5	20.2	40	4	4	None
527L	N Nicotinyl 2 aminopyridine	1.5†	19.0	17.0	10	4	4	None
649L	N Nicotinyl 2 amino 5 isovanisole	0.25	20.2	20.3	50	4	4	None
652L	2 (Nicotinyl amino) phenol	0.25	17.7	20.5	10	4	4	None
653L	3 (Nicotinyl amino) phenol	0.25	17.8	19.7	10	4	4	None
654L	1 (Nicotinyl amino) phenol	0.25	17.8	17.8	10	4	4	None
662L	d,l α Nicotinyl aminobutyric acid	0.25	20.6	17.1	10	4	4	None
729L	p (Nicotinyl amino) benzoic acid	0.25	20.0	20.3	40	4	4	None
725L	2 (N Nicotinyl amino) 5 carbethoxy thiurazole	0.25	18.5	15.6	0	4	4	None
739L	N Methoxyl propyl nicotinamide	0.25	18.5	16.4	10	4	4	None
661L	p (Nicotinyl amino) acetylthide	0.25	20.4	18	20	4	4	None
747L	6 Chloronicotinamide	0.25	18.5	13.5	10	4	4	None
765L	6 Aminonicotinamide	0.1†	19.4					
on 770L	6 Butoxynicotinamide	0.25	20.1	16.1	10	4	1	None
768L	2 Aminonicotinamide	0.25	19.8	16.5	0	4	4	None
769L	1 thyl nicotinamide	0.25	19.0	18.5	10	4	1	None

All the amount of intake were included in the diet at the maximum tolerated concentration  
 \* and at 0.75 mg/ml in the diet and at 0.5 mg/ml in the diet and at 0.25 mg/ml in the diet  
 † at 0.25 mg/ml in the diet and at 0.1 mg/ml in the diet and at 0.05 mg/ml in the diet

lymph nodes were but slightly involved. The infection in the lungs at the time of death was extensive, varying from discrete white nodular patches to extreme consolidation of whole lobes. Microscopically these nodular patches showed varying stages of consolidation, caseation and monocytic and lymphocytic infiltration to a mere proliferation of tissue. Numerous clumps of acid fast bacilli were observed throughout the tubercles.

*Chemotherapy*—The apparent tuberculostatic activity of pyridine carboxylic acid on the chorioallantoic membrane of the chick embryo led us to repeat all of the active compounds in infected mice. For the most part these compounds were fed in the diet. Of this group nicotinic acid and its amide seemed to be the most active compounds. Consequently Kushner and co-workers<sup>9</sup> synthesized a series of thirty derivatives of nicotinamide either in the form of substituents in the acid amide group or as nuclear substitutions. These were tested in mice by the technique already described. The results are given in Table I, but the chemical synthesis and properties of these compounds are published elsewhere.<sup>9</sup> Nicotinamide was the most active chemical tested. All the changes introduced into the nicotinic acid amide molecule either increased the toxicity or reduced the activity or both.

The failure to increase the activity or even to retain the activity of nicotinic acid amide by any slight alteration in the molecule led us to suspect that we might be dealing with the specific activity of a vitamin. Calculating from our results with mice, on a weight basis the therapeutic dose in human beings would be about 100 to 125 grams—a dose which could not be tolerated by the human subject. So other vitamins were tried at 0.1 per cent concentration in the diet with or without a smaller amount of nicotinic acid amide. Of these only riboflavin seemed to have a slight effect in securing the desired end of reducing the total treatment dose of the nicotinic acid amide. See Table II.

In addition, possible naturally occurring precursors of nicotinic acid amide such as tryptophane and 3-hydroxyanthranilic acid were attempted as chemotherapy, but all yielded negative results.

*Resistance of the Tubercle Bacilli to Nicotinic Acid Amide*—One of the possible disadvantages of streptomycin in the treatment of human tuberculosis is that the organisms become resistant to streptomycin in the course of time. Similar resistance studies were made in regard to the possibility of the organisms becoming resistant to the nicotinic acid amide. The organisms were recovered from the mouse previously treated with nicotinic acid amide and cultured on egg medium. The bacterial growth was resuspended and used for infecting the test mice. Five mouse passages showed little evidence of resistance of the tubercle bacilli but later passages showed some evidence of resistance.

However, when the suspension of the lung excised from the mouse that had been treated with a combination of nicotinic acid amide and streptomycin was placed on egg medium such a sparse growth appeared after a prolonged period of incubation that we believe a very much more reduced number of viable organisms existed in the lungs than when streptomycin was administered alone.

TABLE II EFFECT OF TREATING EXPERIMENTAL TUBERCULOSIS OF WHITE MICE WITH NICOTINIC ACID AMIDE IN COMBINATION WITH DIFFERENT VITAMINS

VITAMIN	PER CENT NICOTINIC ACID AMIDE IN DIET	PER CENT ADDITIONAL VITAMIN IN DIET	AVERAGE BEGINNING WEIGHTS (GM.)	AVERAGE WEIGHTS (3 WK.)	PER CENT SURVIVAL (10 MICE PER GROUP)	GROSS AP PEARANCE OF LUNG AT AUTOPSY†	AMOUNT OF DISEASE IN LUNG SECTION†	ACTIVITY‡
None	None	None	17.6	24.1	100	0		
Streptomycin*	None	None	17.8	23.2	100	0	± 1	Active
Streptomycin* plus nicotinic acid amide	0.75	None	19.2	21.6	100	0	± 1	Active
Nicotinic acid amide	0.25	None	18.4	20.9	80	2	2.3	V SI Active
Nicotinic acid amide	0.5	None	18.4	22.6	100	1	2	SI Active
Riboflavin	None	1	19.0	18.8	0	4	4	None
Nicotinic acid amide plus riboflavin	0.25	0.1	20.2	20.9	50	2	2.3	V SI Active
Nicotinic acid amide plus riboflavin	0.25	0.5	19.2	21.2	100	0	1.2	Active
Nicotinic acid amide plus calcium pantothenate	0.1	0.1	19.7	17.9	0	3.4	4	
Nicotinic acid amide plus calcium pantothenate	0.25	0.5	19.0	20.8	30	4	4	None
Nicotinic acid amide plus para aminobenzoic acid	0.1	0.1	19.7	18.7	0	4	4	None
Nicotinic acid amide plus para aminobenzoic acid	0.25	0.1	19.7	18.9	30	4	4	None
Nicotinic acid amide plus inositol	0.1	0.1	18.8	17.9	20	4	4	None
Nicotinic acid amide plus inositol	0.25	0.1	18.8	17.0	10	4	4	None
Nicotinic acid amide plus choline chloride	0.1	0.1	18.9	16.0	20	4	4	None
Nicotinic acid amide plus choline chloride	0.25	0.1	18.8	17.6	30	4	4	None
Nicotinic acid amide plus ascorbic acid	0.1	0.1	20.4	20.3	30	4	4	None
Nicotinic acid amide plus ascorbic acid	0.25	0.1	20.4	20.5	80	4	4	None
Nicotinic acid amide plus mixed tocopherol	0.25	0.1	18.4	16.4	0	4	4	None
Nicotinic acid amide plus vitamins A and D	0.25	A 450 u/day D 105 u/day	18.6	16.2	0	4	4	None
Nicotinic acid amide plus vitamins A and D	0.1	A 450 u/day D 108 u/day	18.9	15.2	0	4	4	None
None (untreated tuberculous mice)			19.2	15.6	0	1	1	

All treatment chemicals were included in the diet at the maximum tolerated concentration

\* Streptomycin, 100 mg/kg body weight, daily for 14 days

## SUMMARY

The oral administration of 0.5 to 0.75 per cent of nicotinic acid amide in the diet will markedly suppress the spread of tuberculosis in experimentally infected mice.

This amount of nicotinic acid amide in the diet was apparently roughly equivalent in activity to injections of 1 mg of streptomycin four times daily over the same test period.

We wish to express our appreciation to Miss Florence Anderson, Miss Barbara Gosford, Mr Samuel Smith, and Miss Hester Snider for their technical assistance.

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# PORPHOBILINOGEN TESTS ON A THOUSAND MISCELLANEOUS PATIENTS IN A SEARCH FOR FALSE POSITIVE REACTIONS

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IN THE last decade acute idiopathic porphyria has been accorded increasing attention as evidenced by more frequent clinical reports of the disease, reviews of the subject, and the description<sup>8</sup> of a new simple diagnostic laboratory procedure. It seemed pertinent to study the Watson and Schwartz porphobilinogen test<sup>8</sup> which is now widely considered as pathognomonic of this disease. The studies reported in this paper consisted of a search for false positive porphobilinogen tests in a large series of patients.

Briefly, porphyria is defined as a primary idiopathic disease, characterized by abnormal amounts of uro- and coproporphyrins and/or porphobilinogen in the urine, due to a derangement of the metabolism of porphyrin compounds. The term porphyria is opposed to porphyrimuria, which refers to abnormal amounts of coproporphyrins in the urine secondary to a variety of diseases such as lead poisoning, liver disease, or the ingestion of drugs (for instance sulfa drugs, Pyramidon or the salicylates). Porphyria is usually divided into two types: the congenital (or light-sensitive) and the acute idiopathic. Congenital porphyria is the rarest type and is characterized by red porphyrin deposits in the bones and teeth (erythrodontia), photosensitivity of the skin (hydra aestivale sen vacciniforme), and a dark-red urine. Acute porphyria occurs in adults with intermittent attacks of acute abdominal crises with cramps and constipation, nerve paralysis and mental symptoms, and frequently a dark urine. The porphobilinogen test (see below) is characteristically positive, while in a small series of cases of congenital porphyria, the porphobilinogen test has been negative according to Watson, Schwartz and Hawkinson.<sup>9</sup>

For years the diagnosis of acute porphyria hinged on the large amounts of uro- and coproporphyrin found in the urine. The excretion of the Waldenström type of uroporphyrin in particular was shown to be increased in acute idiopathic porphyria.<sup>5-9</sup> For a long time the diagnosis of porphyria depended on the chemical identification of uroporphyrin and, to a lesser extent, coproporphyrin, which required a difficult and tedious fractionation. The porphobilinogen test<sup>8</sup> is a simple procedure useful in the diagnosis of acute idiopathic porphyria.

## HISTORY AND PROPERTIES OF PORPHOBILINOGEN AND PORPHOBILIN

In 1931 Sachs<sup>4</sup> first reported evidence of a substance in the urine of a patient with acute porphyria which gave a red color with Ehrlich's reagent (p-dimethyl-amidobenzaldehyde). She reported that this red compound exhibited two spectral absorption bands with Ehrlich's reagent. The red Ehrlich

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aldehyde compound was insoluble in chloroform differentiating it from the urobilinogen aldehyde compound and as shown later by Watson and Schwartz<sup>9</sup> from indole aldehyde compound, as formed with Ehrlich's reagent

Waldenstrom and Vahlquist discovered further properties of this substance<sup>5, 6, 7</sup> They found that if the urine in acute porphyria was exposed to sunlight it became darker and the reaction with Ehrlich's reagent became negative Urea inhibited the development of the reaction Heating the acute porphyria urine with acid caused darkening concomitant with development of a negative Ehrlich's reaction and the appearance of a dark reddish brown pigment which was named porphobilin<sup>8</sup> Some evidence was elicited by Waldenstrom that this pigment was a dipyrromethene

The colorless chromogen of this pigment was designated by Waldenstrom as porphobilinogen A method for measuring porphobilinogen in arbitrary units has been described<sup>6</sup> Neither porphobilinogen nor porphobilin has been chemically isolated or structurally identified Prunty has studied the chemical properties of porphobilinogen and has isolated it from the liver in a patient with acute porphyria<sup>9</sup> This work indicates that it is not a breakdown product in the urine but is formed in the body probably in the liver Further studies are necessary to evaluate its possible role as a precursor of the porphyrin ring

Watson and Schwartz<sup>9</sup> provided the first simple clinical test for acute idiopathic porphyria in 1941 and found it to be positive in all of five cases Others have added confirmatory evidence that the test is at least relatively specific<sup>1, 2, 3</sup>

Watson states<sup>10</sup> that in his laboratories several instances of a false red or pink color have occurred in a porphobilinogen test One was a case of acute polyomyelitis in which the urine contained large amounts of coproporphyrin and urobilinogen In a case of cirrhosis of the liver with large amounts of coproporphyrin in the urine the porphobilinogen reaction was repeatedly although not consistently positive Several urine specimens were found with large amounts of urobilinogen in which it was impossible with repeated extractions to get all of the aldehyde compound into the chloroform The urine from a patient with jaundice showed a positive porphobilinogen test in which the red color behaved like an indicator with variations of pH the red color disappearing with alkalinization In this instance the red color was thought to be due to an extrinsic pigment perhaps a drug Clayons and beets ingested by infants gave weak but definitely positive porphobilinogen reactions Watson now considers the test "in the main very helpful and reliable for porphyria but I do not believe the test can be spoken of as absolutely specific or pathognomonic"

#### METHODS AND MATERIALS

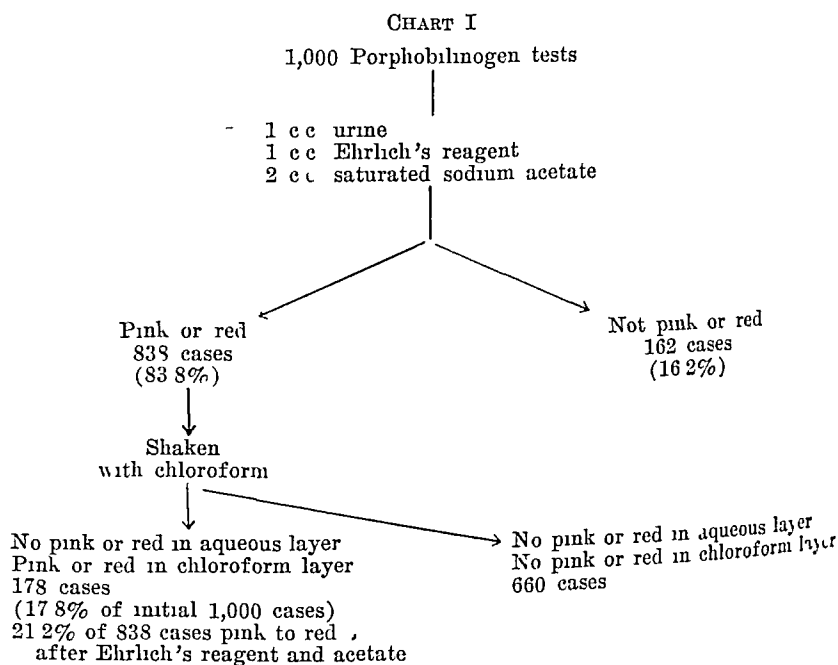
The purpose of the present study was to evaluate this simple office procedure in regard to false reactions There is no conclusive method for ruling out false positive reactions with any simple test except by means of large scale examination of patients with miscellaneous diseases The tests were done in the early morning with fresh urine and from ten to thirty five were done each day Urine specimens of 1000 different patients were examined by the porphobilinogen test of Watson and Schwartz in which 1 ml. of Ehrlich's reagent (0.7 Cm

p-dimethyl-amidobenzaldehyde, 150 ml concentrated HCl, and 100 ml distilled water) was mixed with 1 ml of urine, and then 2 ml of a saturated aqueous solution of chemically pure sodium acetate also were added and mixed. If any pinkish or reddish color resulted, a few milliliters of chloroform were added and the whole was forcibly shaken in a stoppered tube.

The 1,000 patients on which the study was based came from the medical, surgical, obstetric, gynecologic, urologic, orthopedic, neurological, eye, ear, nose, and throat services of the Worcester City Hospital.

#### RESULTS

No positive porphobilinogen tests were obtained. In no case did a pinkish or red color remain in the aqueous layer when the test was performed properly. It is important to call attention to three points in particular: (1) The chloroform extraction requires a very thorough shaking with the aqueous fraction. (2) Red-colored globules of chloroform frequently adhere to the sides of the



tube in the upper portion giving a false color to the aqueous layer. (3) Reflection of the reddish color from the lower (chloroform) layer into the aqueous layer must be discounted. The authors have been consulted by clinicians who were confused by what purported to be a positive porphobilinogen test in which one of the errors mentioned had occurred. The necessity for these precautions is emphasized by the fact that 838 (83.8 per cent) of the 1,000 cases showed some pink color in the aqueous layer prior to the addition of chloroform. In the majority of the 838 cases the color was a faint pink and in these all that remained after the addition of chloroform was a yellow or slightly orange tint in the chloroform layer. In 21 per cent of the 838 cases the chloroform layer became pink or red after the extraction procedure. The distribution of the reactions is represented by Chart I.

## SUMMARY

The simple porphobilinogen test as described by Watson and Schwartz was studied by the examination of the urine of 1,000 patients

No positive reactions were found. From this it may be concluded that false positive reactions are extremely rare, and that the test has a great deal of significance in the diagnosis of porphyria.

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# HEPATIC DYSFUNCTION IN INFECTIOUS MONONUCLEOSIS, WITH REVIEW OF THE LITERATURE

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THE following paper is a report of the incidence of hepatic dysfunction in a group of hospitalized patients with infectious mononucleosis, with a review of the literature on the subject of liver involvement in infectious mononucleosis.

## REVIEW OF LITERATURE

Pfeiffer,<sup>1</sup> in his original description of the disease called glandular fever, mentioned enlargement of the liver as one of the physical findings. Snapper and co-workers<sup>2</sup> were the first to describe jaundice as a complication of glandular fever. Downey and McKinlay<sup>3</sup> in 1923 were the first observers in this country to describe jaundice in a patient with infectious mononucleosis. Later, in 1930, McKinlay<sup>4</sup> reported a series of fifty cases with jaundice in 10 per cent. McKay and Wakefield<sup>5</sup> in 1926 reported one case of infectious mononucleosis with jaundice. Schmidheiny<sup>6</sup> in 1927 reported on eleven cases, with icterus in four. Mason<sup>7</sup> in 1928 reported two cases with jaundice and suggested that the jaundice was the result of a hepatitis, rather than the result of pressure of enlarged nodes on the common bile duct as had been suggested by McKay and Wakefield.

Nyfeldt<sup>8</sup> reported a series of thirty-three cases with hepatomegaly in nine and icterus in four, and in 1934 Stuart and co-workers<sup>9</sup> reported a series of twenty-eight cases with jaundice in two. Farley<sup>10</sup> reported twelve cases with hepatomegaly in four, clinical jaundice in one, and bilirubinuria in an additional one. DeVries<sup>11</sup> reported three cases with clinical jaundice in 1938. Paul,<sup>1</sup> in a review of infectious mononucleosis in 1939, reported an incidence of jaundice in 10 per cent of fifty cases. In a later paper Gardner and Paul<sup>12</sup> reviewed 137 cases at the New Haven Hospital and found jaundice in only five per cent, however, 13 per cent had hepatomegaly.

Svaen-Seljesaeter,<sup>14</sup> Fowler and Tidrick,<sup>15</sup> Bernstein,<sup>16</sup> Gold,<sup>1</sup> Howard Carter,<sup>19</sup> Boger,<sup>20</sup> Monat,<sup>21</sup> Leavell and McNeal,<sup>22</sup> and Wising<sup>3</sup> each described one case with jaundice. Martin<sup>24</sup> reported two cases with jaundice, and Chapman and Chapman<sup>25</sup> reported seven cases with mild clinical jaundice.

Ollgaard<sup>26</sup> reported a large series of 210 cases with icterus in four. Ottenberg and Spiegel<sup>27</sup> reported sixty-seven cases with jaundice in two. Contratto<sup>1</sup> 196 cases with ten jaundiced, Immerman,<sup>23</sup> 220 cases with three jaundiced, Milne,<sup>30</sup> 111 cases with three jaundiced, Read and Helweg,<sup>31</sup> 300 cases with jaundice in eleven but with forty-seven showing hepatomegaly. Spring<sup>22</sup> described the clinical picture in five cases with an elevated icteric index. Pres-

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and co workers<sup>33</sup> reported on a series of ninety six cases with jaundice as the presenting complaint in five, though the liver was enlarged in twenty six of the patients

Recently Abrams<sup>34</sup> reported one case with jaundice of eleven weeks duration He also mentions an incidence of jaundice of 9.4 per cent in a series of sixty four cases

Halcrow and associates<sup>35</sup> in a report of an epidemic of infectious mononucleosis in a hospital in England found no clinical jaundice in 296 cases However, only 125 of the patients had clinical symptoms whereas the other 169 had only blood and/or serologic changes They studied fifteen of the more severe cases selected at random and found latent jaundice as manifested by a slightly elevated serum bilirubin, in eight

In a recent report of an epidemic extending over a fifteen month period at an Army post, Wechsler and Rosenblum<sup>36</sup> in the largest series of cases of infectious mononucleosis ever reported (556 cases) found thirty five cases (7.9 per cent) with jaundice Most of the cases were mild as far as the icteric index and gastrointestinal symptoms were concerned They found that the liver was palpable in ninety five of the 556 cases however they did liver function tests only in those that showed clinical jaundice Liver function tests in many of these patients showed an elevated direct reacting bilirubin positive cephalin cholesterol test elevated serum alkaline phosphatase increased bromsulfalein retention, and elevated urine urobilinogen In none of the patients that showed jaundice were there any sequelae

Cohn and Lidman<sup>3</sup> reported on a group of fifteen successive patients hospitalized at an Army hospital for infectious mononucleosis None of the fifteen were jaundiced clinically however by means of serial hepatic function studies they were able to demonstrate evidence of hepatic dysfunction in all fifteen DeMarsh and Alt<sup>38</sup> reported nineteen consecutive cases of infectious mononucleosis without jaundice (icteric index less than 10) of which all showed either positive cephalin cholesterol delayed bromsulfalein excretion or reversal of the albumin globulin ratio Gall<sup>39</sup> did liver function tests on thirty three cases serially and found an elevated alkaline phosphatase in all but five cases twenty two of twenty six had positive cephalin cholesterol tests fourteen of fifteen had an elevated thymol turbidity and nine of twenty showed an elevated icteric index (above 8 units) Evans<sup>40</sup> did liver function tests on nineteen consecutive cases without jaundice (thirteen hospitalized and six ambulatory) He found the thymol turbidity elevated in 68 per cent cephalin cholesterol positive in 95 per cent, alkaline phosphatase increased in 43 per cent and in three cases in which electrophoretic determinations were done on the serum he found an increased amount of beta and gamma globulin

Shry and associates<sup>41</sup> found an elevated thymol turbidity and positive cephalin cholesterol and serum colloidal gold tests in some cases of infectious mononucleosis Carter and MacLagan<sup>42</sup> found a positive colloidal gold reaction in 9.1 per cent and an elevated thymol turbidity in 58 per cent of nineteen patients with infectious mononucleosis but did not feel that this necessarily indicated any hepatic disturbance

Kilham and Steigman<sup>43</sup> were the first observers to demonstrate, by direct observation of the liver cells, that the jaundice in patients with infectious mononucleosis was the result of a hepatitis. In their series of twenty patients there were four clinically jaundiced, whereas others had slight hyperbilirubinemia as demonstrated by laboratory tests. A liver biopsy of one of the jaundiced patients showed a well-marked focal hepatitis. They found maximal changes in the portal tracts with loss of liver cells and a well-developed histiocytic reaction with some early proliferation of bile ducts. The sinusoids showed an excess of Kupffer cells and monocytes. They found no evidence of fibrosis. Van Beek and Haex<sup>44</sup> did liver biopsies on a patient with infectious mononucleosis without jaundice and described the changes as similar to those seen in patients with myeloid leukemia with many monocytoïd cells and neutrophils. A few of the cells showed mitosis. They did a repeat biopsy on the same patient three weeks after recovery and found the liver to be entirely normal. Bang and Wanscher<sup>45</sup> did aspiration biopsy on four patients with infectious mononucleosis with jaundice, however, only two of the four had enlarged livers. All of the sections showed pronounced infiltration of the portal areas with lymphocytes, and a few plasma cells, neutrophils, and eosinophiles. A few of the liver cells showed mitosis, though no necrosis was evident. There was no evidence of bile duct proliferation or fibrosis. It was their impression that the changes were very similar to those seen in acute epidemic hepatitis with, however, less pronounced parenchymatous changes and more interstitial changes.

Ziegler,<sup>46</sup> Davis and co-workers,<sup>47</sup> Fisher,<sup>48</sup> Allen and Kellner,<sup>49</sup> and Peters and associates<sup>50</sup> reported on the histologic picture of the liver of patients with infectious mononucleosis without jaundice or hepatomegaly who were dying of some other cause. The patients of the first three observers had all died of spontaneous rupture of the spleen. In these cases numerous small foci of infiltrations of mononuclear cells were found. Within these foci there was some degeneration of parenchymal cells. Allen and Kellner's patient had been killed in an airplane accident about a week following recovery from infectious mononucleosis. They, also, found many focal areas of infiltrations of mononuclear cells, which were mostly perlobular in distribution. Some of the liver cells showed mitosis. Peters' two patients died as a result of complicating Guillain-Barré's disease. They describe the liver of one of these patients as showing the picture of a moderate hepatitis.

#### MATERIALS AND METHODS

The present series consisted of forty consecutive patients with infectious mononucleosis admitted to the Students' Health Service\* over a fifteen month period of time in 1946-1947. This does not include patients with infectious mononucleosis of a mild nature that were cared for on an outpatient basis. Also, no patients were included in this group whose clinical criteria and laboratory tests were unequivocal for infectious mononucleosis. Patients at some time during the illness had an absolute lymphocytosis with atypical leucocytoïd lymphocytes and a heterophile titer of 1:112 or higher. Of course it is recognized that a small percentage of patients never develop a positive heterophile titer.

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Heterophile determinations were done according to the method of Davidsohn.<sup>1</sup> A titre of 1:112 was considered as definitely positive. Determination of the prompt direct one minute and total bilirubin was made by means of the Ducci and Watson<sup>2</sup> modification of the Malloy and Evelyn<sup>3</sup> method. Values of greater than 0.2 mg per 100 cc of serum for the prompt one minute direct and values of greater than 0.8 mg per cent for the total delayed direct and indirect reacting bilirubin were considered elevated. The thymol turbidity test was performed according to the technique recommended by MacLagan.<sup>4</sup> Values greater than 4 units were considered elevated. The thymol flocculation was done according to the technique of Neefe.<sup>5</sup> Values greater than 1+ were considered abnormal. The cephalin cholesterol flocculation test was performed according to the method of Hanger,<sup>6</sup> and values greater than 1+ at twenty four hours were considered abnormal. The bromsulfalein test was performed according to the method described by Gaebler,<sup>7</sup> using 5 mg of the dye per kilogram of body weight. The presence of more than five per cent of the dye remaining after forty five minutes was considered abnormal. The alkaline phosphatase was done according to the method of Bodansky<sup>8</sup> as modified by Alessandri and Ducci.<sup>9</sup> Values greater than 40 Bodansky units were considered elevated. Total cholesterol and cholesterol esters were determined according to the method of Sperry and Schoenheimer.<sup>10</sup> A total cholesterol greater than 220 mg per 100 cc of serum and/or a depression of the esters to less than 60 per cent of the total was considered abnormal. The estimation of the urinary Ehrlich reaction was made according to the methods of Watson and co-workers.<sup>11, 12</sup> Ehrlich units of greater than 1.4 per two to four hour specimen were considered abnormal and excretion of more than 3.5 mg of urobilinogen per day was considered abnormal. The urinary coproporphyrin was determined according to the method of Schwartz and associates,<sup>13</sup> and the excretion of more than 100 gamma per day was deemed abnormal.

## RESULTS

Of the forty patients with unequivocal evidence of infectious mononucleosis included in the present study, twenty one (Cases 1 to 21, Table I) showed clear cut signs of hepatic functional impairment as evidenced by three or more positive liver function tests exclusive of the test for urinary coproporphyrin. A few of the remaining nineteen patients (Cases 22 to 40, Table I) showed questionable evidence of hepatic dysfunction on the basis of one or two mildly positive tests. The frequency of the abnormality of the various tests in the forty cases is shown in Fig. 1.

Only one of the patients with hepatic functional impairment had definite jaundice, and only four of the twenty one had an enlarged liver. In three of these four it was definitely tender, and there was tenderness in the liver region in one other patient of this group in whom it was not possible to feel the liver. Forty-eight per cent of the group with hepatic functional impairment had splenomegaly which is about the same (53 per cent) as the group without hepatic dysfunction. All of the patients without laboratory evidence of hepatic dysfunction had a definite pharyngitis whereas three of the twenty one patients with marked hepatic dysfunction had no pharyngitis at any time and three additional patients only developed pharyngitis late in the course of the disease when the hepatitis had nearly subsided. The febrile course did not appear to be greatly different in those with or without hepatic dysfunction. The average maximum temperature elevation in the group with hepatitis was 101.7 degrees F and in the other group, 101.5 degrees F.

Case 22 is of interest in that the patient returned about one week after discharge from the hospital with a complaint of recurrence of malaise, and





	1-11	04	11	6	3	4	1-	99	64	16-	5-	1448	67	+
18	1-11	04	11	6	3	4	1-	99	64	16-	5-	1448	67	+
19	5/-	04	05	5	2	4	10	27	123	146	68	1234	74	0
20	1/15	01	08	7	Tr	3	6	27				1448	85	+
21	1/15	01	07	4	Tr	2	6	04						
22	1/8	02	10	1	0	1+	19	28						0
23	1/10	03	14	-	0	2	36	325	739	162	51			
24	1/15	01	39	6	0	4	36	62	115					
25	1/21	05	12	5	Tr	3		36	91	239	182			
26	4/-6	01	09	1	0	0	5	14	133			1896	62	+
27	5/23	01	07	9	2+	1	2	04				1792	76	+
28	10/8	00	05	2	0	0	5	02						
29	10/11	01	06	-	2	3	3	09				1448	63	+
30	2/30	01	04	-	1	1	1896	59				1896	59	+
31	4/19	01	03	4	0	2	2	05	45	32		1192	68	+
32	4/14	02	12	3	0	1	4	04	09	204	69	1448	56	+
33	10/5	01	09	2		2	4	04	08	40		1448	59	+
34	10/11	01	06	1		3	7	07	73			1448	81	+
35	10/10	01	06	4		2	9	10				1448	60	+
36	10/8	00	05	3		1	4	30				1596	61	+
37	2/8	01	05	3		1		05	03	46		1448	62	+
38	5/1	01	04	4	Tr	Tr		05	29	136		1244	65	+
39	6/9	01	04	2	Tr	Tr			47			1924	58	+
40	10/8	01	04	7		4	4	06	44	11		1192	64	+
41	10/8	01	04	4		1	4	06	07	6		1896	60	+
42	5/9	01	04	3	Tr	2	4	01	18	4-		1448	64	+
43	9/5	01	04	6		2	C	02				1448	63	+
44	1/23	01	00	4	0	1	5	1				1244	63	+
45	3/2	02	06	3	0	1	5		38	147		1896	71	+
46	3/2	02	06	3	0	1	5		38	147		1792	58	+

1 B One minute, or prompt reacting serum bilirubin (mg/100 cc.) TB delayed direct and indirect reacting serum bilirubin (mg/100 cc.) TT thymol turbidity (30 min reading) TF thymol flocculation (1+ to 4+ at 18 hr) CC cephalin cholesterol flocculation (1+ to 4+ at 24 hr) BSP bromsulphalein retention (45 min after mg/kg body weight) U/L urobilinogen in urine (Ehrlich units 2 to 4 hr) UU urobilinogen in urine (mg/day) UC coproporphyrin in urine (gamma/day) AP alkaline phosphatase (Bodansky units/100 cc serum) TC total serum cholesterol per 100 cc serum cholesterol esters (per cent of total cholesterol) HT heterophilic antibody titre, L, highest per cent of lymphocytes in a total white blood cell count above 6000 LL enlarged liver TL tender liver P pharyngitis and/or tonsillitis

Developed pharyngitis late in course of disease after subsidence of hepatitis

relaxa, and nausea Examination showed the liver edge to be palpable at the costal margin and moderately tender This picture, along with the elevated thymol turbidity and the positive thymol flocculation test, may have indicated a relapse with a hepatitis, however, the patient quickly improved on a regime of restricted activity

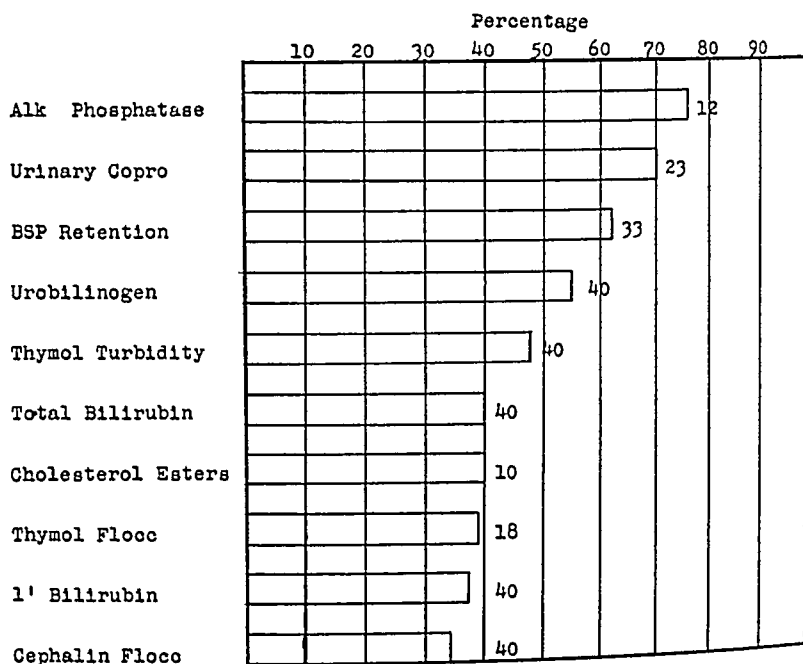


Fig 1—Percentage of liver function tests that were positive in a group of forty patients with infectious mononucleosis The numbers at the ends of the bars indicate the number of patients on whom each test was performed

In none of the group of forty patients were there any serious complications. In the group with hepatic dysfunction, the liver function tests showed a return to normal in a short period of time In general the patients with hepatic dysfunction appeared to have a slightly more severe form of disease

The patients with obvious hepatic involvement were placed on a high protein, high carbohydrate diet, whereas the others were maintained on a general hospital diet Otherwise the therapy was purely supportive with bed rest when indicated by the temperature and degree of hepatic dysfunction, except for the use of penicillin in a few patients who appeared to have developed an exudative pharyngitis or tonsillitis as a complication

#### DISCUSSION

For years there has been a debate in the literature about the possible cause of the jaundice in patients with infectious mononucleosis Most of the earlier papers mentioning jaundice as a manifestation of infectious mononucleosis suggested that it was the result of pressure of enlarged lymph nodes on the common bile duct, and was thus an obstructive jaundice<sup>3, 21, 24, 42</sup> However, "

early as 1928<sup>7</sup> it was suggested that the jaundice was the result of a hepatitis. With the advent of the use of numerous liver function studies for aids in differentiating obstructive and nonobstructive jaundice it became possible to resolve this question. A number of investigators<sup>30-40</sup> have shown that patients with infectious mononucleosis have the same kind of abnormalities in the liver function tests as patients with epidemic or sporadic infectious hepatitis and homologous serum hepatitis. This work has all pointed to the presence of a hepatocellular and cholangiolar type of liver injury rather than an extrahepatic obstruction as the cause of the jaundice. It also has been found that the general serum protein disturbances are characteristically the same in infectious mononucleosis as those found in infectious hepatitis; that is, a slight decrease in the albumin and a marked increase in the beta and gamma globulins.<sup>3-40</sup>

The presence of an intrahepatic cause for the jaundice in these patients received more definitive proof with the histologic demonstration of hepatitis as a result of punch biopsies of the liver of patients with infectious mononucleosis with jaundice<sup>43-4</sup> and in a patient without jaundice<sup>44</sup> and also by the demonstration of hepatitis microscopically in patients with infectious mononucleosis without jaundice on autopsy sections of the liver.<sup>45-6</sup>

The present study of forty cases of infectious mononucleosis also demonstrates clearly the presence of both hepatocellular and cholangiolar liver damage as the basis for the functional impairment of the liver and in one case (Case 8) for clinical jaundice. Case 16 was of interest in that the patient exhibited predominately a cholangiolar type of hepatitis; namely, increased prompt reacting (one minute) bilirubin, alkaline phosphatase, total cholesterol and the presence of an intense pruritis.

Thus it would appear that even though jaundice is not especially common in infectious mononucleosis, hepatitis without jaundice is extremely common. The criticism might be ventured, however, that this group of patients is not particularly representative of patients with infectious mononucleosis and that the degree of liver involvement is out of proportion to that seen in the usual hospitalized patient with infectious mononucleosis. Yet the percentage of patients in this series with jaundice (2.5 per cent) is no higher than reported by other investigators: 10,<sup>4</sup> 12,<sup>8</sup> 7,<sup>5</sup> 5,<sup>13</sup> 4,<sup>23</sup> 2,<sup>28</sup> 3,<sup>27</sup> 5,<sup>28</sup> 14,<sup>29</sup> 7.7,<sup>30</sup> 3.3,<sup>31</sup> and 7.5 per cent.<sup>32</sup> The percentage of patients with hepatomegaly might also give some clue as to the probable incidence of hepatic involvement in other reported series. It was 15 per cent in the present group of forty cases. Others have variously reported the incidence at 27,<sup>8</sup> 33,<sup>10</sup> 13,<sup>13</sup> 16,<sup>31</sup> 26,<sup>33</sup> and 17 per cent.<sup>30</sup>

It is fair to assume that one would anticipate more patients with hepatitis without jaundice than patients with jaundice. It has been demonstrated in the development of experimental hepatitis in human volunteers that the number of patients that develop hepatitis without jaundice exceeds the number that develop jaundice.<sup>44</sup>

The criticism might be anticipated that one could get the degree of hepatic involvement seen in these cases in simple upper respiratory infections or cer-

tain relatively benign viral infections. However, it has been shown by others,<sup>64</sup> that such is not the case, nor have I observed this degree of functional impairment of the liver in the pharyngitides and upper respiratory infections. Also, four of these patients with hepatitis had no pharyngitis at any time and very little fever, and three others did not develop the pharyngitis until after the hepatitis had nearly subsided. For hyperthermia alone to cause a marked degree of liver functional impairment it is necessary for the patient to be exposed to a severe and prolonged hyperthermia.<sup>65, 66</sup>

A patient with acute infectious mononucleosis and hepatitis and a patient with acute epidemic or sporadic infectious hepatitis often pose a difficult problem in differential diagnosis. Clinically the one symptom that is most valuable in differentiating the two conditions is sore throat. Most all patients with infectious mononucleosis will complain of a moderate to severe sore throat during some period of the illness, whereas this is not a very outstanding sign in infectious hepatitis. As far as physical signs are concerned this is also a valuable guide. There were only four patients out of the entire group of forty that did not have a definite pharyngitis at some stage of illness. All four of these were in the group with definite hepatic dysfunction. This pharyngitis is usually of the nonexudative, nonspecific type, but in some cases is of the exudative type and beta hemolytic streptococci can be isolated from the throat. Lymph node enlargement is distinctly more common and more marked in infectious mononucleosis than in infectious hepatitis, however, the difference is not too striking. Various investigators have listed splenomegaly in infectious hepatitis as 20,<sup>67</sup> 13,<sup>68</sup> and 21 per cent.<sup>68</sup> In our cases of infectious mononucleosis we have found splenomegaly in 48 per cent, which is comparable to what other observers have found: 50,<sup>4</sup> 47,<sup>28</sup> and 34 per cent.<sup>31</sup>

Conversely, the liver is probably much more often enlarged in infectious hepatitis than in infectious mononucleosis. However, Zimmerman and co-workers<sup>68</sup> found the liver to be enlarged in only 69 per cent of a series of patients with infectious hepatitis in which 90 per cent of the patients were jaundiced. There are few papers on infectious hepatitis without jaundice, so it is difficult to make a comparison with patients with infectious mononucleosis without jaundice. Barker and associates<sup>64</sup> and Finks and Blumberg<sup>69</sup> state that in infectious hepatitis without jaundice the liver is usually enlarged. Fifteen per cent or six of the forty patients in this group showed hepatomegaly, and four of these were in the group with hepatitis. Others have reported similar figures: 16 per cent,<sup>31</sup> and 17 per cent.<sup>36</sup>

Thus it may be seen that there is considerable overlapping in the clinical picture in these two conditions, and in the final analysis one must rely on laboratory tests to differentiate the two. Patients with both infectious hepatitis and infectious mononucleosis show the atypical or leucocytoid lymphocytes of Downey,<sup>70</sup> Types I, II, and III—mostly Type II. However, patients with infectious mononucleosis have a much greater number and at some stage in the disease practically always have an absolute lymphocytosis with large numbers of leucocytoid lymphocytes, whereas patients with infectious hepatitis only de-

velop a relative lymphocytosis. It has been found that in experimentally induced infectious hepatitis in human beings there is only a relative lymphocytosis with the greatest number of atypical lymphocytes at the fourth to fifth day after the onset of fever.<sup>71</sup> In infectious mononucleosis the lymphocytosis usually is not transient but increases in degree as the disease progresses and usually remains absolute after the symptoms have subsided.

The heterophilic antibody titer is equally as helpful as the lymphocytic reaction in differentiating the two conditions. It is practically always positive in infectious mononucleosis if enough determinations are obtained whereas we have never found it to be positive in infectious hepatitis. Others also have been unable to find an elevated heterophile titer in infectious hepatitis.<sup>3, 33, 40, 49, 64</sup> Laton and associates<sup>72</sup> noted a moderate increase in titer of antibodies to sheep red blood cells in some patients with infectious and homologous serum jaundice. These antibodies, however, were of true Forssman type adsorbed by guinea pig kidney, thus unlike those of infectious mononucleosis.

From the present study it is apparent that liver function tests are of little value in differentiating infectious hepatitis from infectious mononucleosis with hepatic functional impairment. The liver profiles of the patients with infectious mononucleosis are for the most part characteristic of those seen in infectious hepatitis. Fig 1 shows graphically the tests that were most frequently positive in the patients with infectious mononucleosis and in general these are the same tests that are positive in the greatest percentage in patients with infectious hepatitis.

The urinary coproporphyrin excretion is increased in both diseases but is of no value in differentiating the two because the increase in both is of the Type I isomer. This is considered in more detail elsewhere.<sup>3, 74</sup>

In this series of patients with infectious mononucleosis as in patients with infectious hepatitis<sup>7, 70</sup> the increase of serum bilirubin is due to an increase of the prompt reacting type more than the delayed or indirect. Evans<sup>40</sup> found the cephalin cholesterol test a more sensitive indicator of hepatic dysfunction than the thymol turbidity test in infectious mononucleosis. In this series the reverse has been found to be true. Also the thymol test remains positive longer than any of the other tests, this also has been found to occur in cases of infectious hepatitis.<sup>6, 7</sup> In the present series there is likewise shown to be a better agreement between the thymol flocculation and cephalin cholesterol flocculation than between the thymol turbidity and thymol flocculation. This is contrary to what others have found in infectious hepatitis.<sup>70</sup> It also has been reported as unusual for the thymol turbidity test to be positive in the absence of a positive thymol flocculation test in infectious hepatitis.<sup>6</sup> However that has not been the case in the present group of cases of infectious mononucleosis.

#### SUMMARY

A series of forty cases of typical infectious mononucleosis has been studied in which twenty one (55 per cent) of the patients showed moderate to severe hepatic functional involvement or hepatitis as evidenced by a battery of liver

function tests. A number of additional patients in the group exhibited milder hepatic involvement on the basis of one or two positive tests.

It has not been found feasible to differentiate the type of liver involvement in these patients with infectious mononucleosis and hepatic dysfunction from that in cases of acute epidemic or sporadic hepatitis. One must rely on the clinical picture, especially the presence of pharyngitis, the type of lymphocytic reaction, and the titer of the heterophile antibodies. The character of the liver function disturbances in these patients points to the presence of both hepatocellular and cholangiolar liver injury. This evidence together with the histologic findings reported in the literature tends to refute the theory that jaundice in infectious mononucleosis is due to an extrahepatic obstruction of the common bile duct by enlarged lymph nodes.

The literature on the subject of hepatic involvement in infectious mononucleosis is reviewed.

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## FOLIC ACID METABOLISM STUDIES

### III INTRAVENOUS ADMINISTRATION OF PTEROYLGLUTAMIC ACID AND PTEROYLTRIGLUTAMIC ACID

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WITH THE TECHNICAL ASSISTANCE OF FRANCES PANZER AND PATRICIA SPARKS

RECENT studies<sup>1</sup> revealed that conjugases present in blood were capable of releasing folic acid from combined forms of the vitamin in blood or from pteroylheptaglutamic acid. This was evidenced by an increase in the folic acid content obtained when blood from various animals including man was incubated at pH 7.0 as compared with the values obtained before incubation.

Considerable interest has developed in determining the comparative effectiveness of folic acid and related compounds in the treatment of macrocytic anemias and related diseases. Excretion studies conducted with normal subjects have shown that negligible amounts (less than 10  $\mu$ g per day) are excreted in the urine when average diets are ingested. The urinary excretion of the vitamin is markedly increased however when oral or intravenous supplements of pteroylglutamic acid or its conjugates are administered. The major portion of the dose is excreted in the first twenty-four hours.

The effect of the amount of folic acid ingested on the folic acid content of the tissues, including blood, has been investigated with various animal species.<sup>2,12</sup> The apparent free folic acid in the blood of the turkey, for example, is markedly reduced when a diet low in the vitamin is given.<sup>11,13</sup>

In the present work the effect of administering folic acid intravenously either as pteroylglutamic acid or as pteroyltriglutamic acid to human subjects on the blood levels of the vitamin was determined at different time intervals after injection. These studies have afforded information on the comparative effectiveness of the two forms of the vitamin in maintaining the blood level and evidence for the rapid cleavage of pteroyltriglutamic acid to compounds active for the test organism *Streptococcus faecalis* R.

#### EXPERIMENTAL

Normal adult human beings consuming average diets were used in this study. Six subjects were used in each of two series of tests. Three subjects in each series received pteroylglutamic acid and three received pteroyltriglutamic acid. Two of the three subjects in each group also were used in the second series conducted two weeks later and the test compound administered was the reverse of that given in the first series. Blood samples

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We are indebted to Dr. T. H. Jukes, Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y., for suggesting this problem and for generous supplies of pteroylglutamic acid and pteroyltriglutamic acid. The cooperation of R. L. Simpson, George Barron, Carl Wilmer, and Sparks, Juanita Pou, Dr. R. T. Holman, and the staff member of the College Hospital in the course of these studies is greatly appreciated.

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were taken by vein before injection of the test compound and two, four, eight, and twenty four hours later Pteroylglutamic acid at a level of 12 mg or the equivalent on a molar basis of pteroyltriglutamic acid was injected intravenously

Appropriate dilutions of the oxalated whole blood from each subject at each time interval were made. The blood samples were autoclaved to inactivate the blood conjugases and prepared for assay in the usual manner. The folic acid content was determined after each treatment with *S. faecalis* R as the test organism<sup>14</sup>. In one of the series, comparative results were obtained when *Lactobacillus casei*<sup>14</sup> was used as the test organism. The amount of folic acid per milliliter of blood is shown graphically in Fig 1 for the *S. faecalis* R assays and in Fig 2 for the *L. casei* assays. The individual values for each subject obtained at each time interval are also indicated.

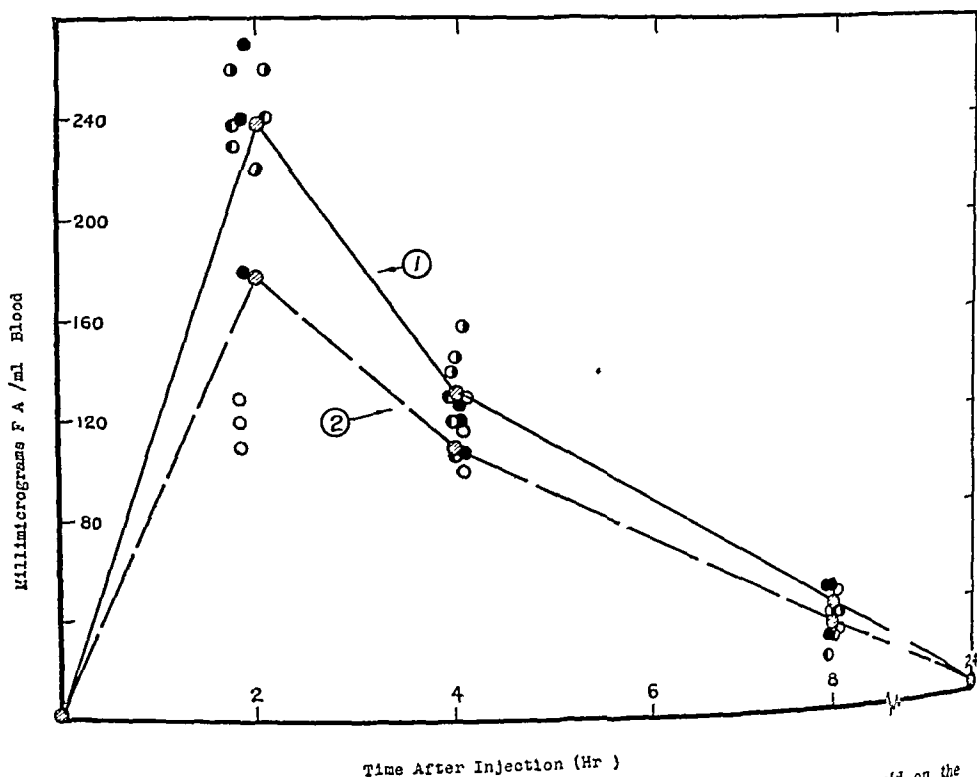


Fig 1—Effect of administering pteroylglutamic acid or pteroyltriglutamic acid on the level of folic acid in the blood measured with *S. faecalis* R as the test organism. 1, Pteroylglutamic acid. 2, Pteroyltriglutamic acid. ① ① first series. ② ② second series. The crosshatched circles represent the averages for the six tests conducted at each time interval.

## RESULTS AND DISCUSSION

The folic acid of the blood as determined includes only those compounds that have activity for the test organisms without treatment of the sample. Pteroylglutamic acid is equally active for both microorganisms. Pteroyltriglutamic acid is essentially inactive for *S. faecalis* R but is quite active for *L. casei*.<sup>2</sup> Therefore in order for pteroyltriglutamic acid to elicit a response with *S. faecalis* R, this vitamin derivative must be cleaved to pteroylglutamic acid or other derivatives which can be utilized by *S. faecalis* R. For *L. casei*, how

ever, both test compounds are active and cleavage of pteroylglutamic acid is not necessary for microbiologic activity. On the other hand if pteronic acid is formed it would be essentially inactive for *L. casei* but active for *S. faecalis* R.

It will be noted that the level of the free vitamin in the blood as determined with both test organisms is very low before injection and that it returned to this low level twenty four hours after injection. For many of the subjects the free folic acid level was too low to measure ( $<0.6$  millimicrograms per millimeter). A prompt and rapid rise in the blood levels as determined with

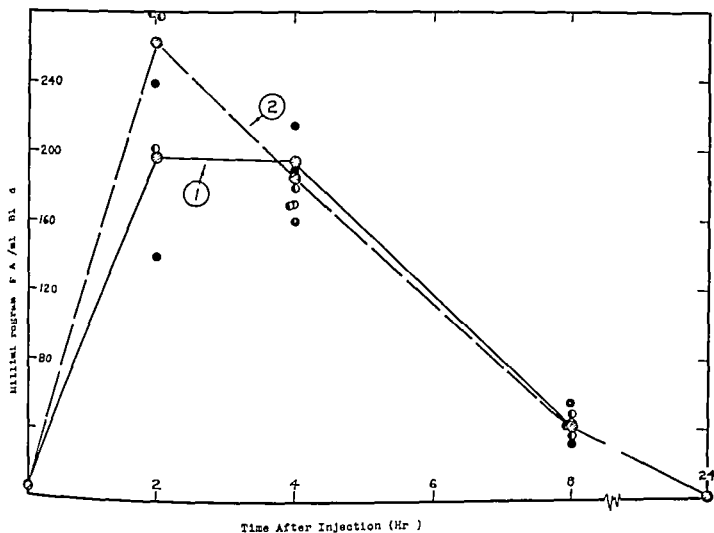


Fig. 2.—Effect of administering pteroylglutamic acid or pteroyltriglutamic acid on the level of folic acid in the blood measured with *L. casei* as the test organism. 1 Pteroylglutamic acid ●● first series 2 Pteroyltriglutamic acid ○○ first series. The cross hatched circles represent the averages for the three tests conducted at each time interval.

*S. faecalis* R occurred after injection and was at a maximum two hours after injection with a decrease observed in the blood levels four and eight hours after injection (Fig. 1). The data obtained with *L. casei* (Fig. 2) indicate a high level of the vitamin at two and four hours after injection with a rapid fall occurring at eight hours after injection\*.

The values obtained for the different subjects at each time interval are in rather good agreement when *S. faecalis* R was used as the test organism with the exception of those obtained two hours after pteroyltriglutamic acid

The data do not permit a definite statement as to the time after injection when the maximum blood levels were reached but are discussed as comparisons at the intervals when analyses were made. The data do show that the maximum blood levels were reached within four hours after the injection of both vitamin derivatives as measured with *S. faecalis* R.

was injected. The values obtained in the second series appear less reliable since they were calculated from data obtained from the low portion of the standard curve and since they do not indicate clearly a decrease when compared with the data obtained four hours after injection. At any rate, both compounds were effective in raising the blood level of folic acid, and pteroyltri-glutamic acid was utilized essentially as well as pteroylglutamic acid. The over-all response was essentially the same for each subject, and the same was true whether the subject received pteroylglutamic acid in the first or second series of tests.

It is also apparent that conjugases presumably of the blood and possibly from other tissues were highly effective in splitting pteroyltri-glutamic acid into products that were highly active for *S. faecalis* R. The ability of conjugases from human blood to release active components from pteroylhepta-glutamate has been described in a previous paper.<sup>1</sup>

The values obtained with *L. casei* before injection and twenty-four hours thereafter were in essential agreement with those obtained with *S. faecalis* R. The values obtained four hours after injection were usually higher with *L. casei* as the test organism, particularly when the tri-glutamate was administered, suggesting that some of the injected tri-glutamate was not cleaved for as long as four to eight hours after injection. The over-all curves for the values obtained with the two organisms when either pteroylglutamic acid or pteroyltri-glutamic acid was administered correlate very well however (Figs 1 and 2).

It is of interest that an increase in the urinary excretion of folic acid following the ingestion of pteroylglutamic acid or pteroyltri-glutamic acid also occurred only for the first twenty-four hours after administration. These techniques should be valuable for further studies not only on the metabolism of pteroylglutamic acid and related compounds by normal human beings, but also on the metabolism of these important nutrients by patients with diseases that respond to treatment with folic acid and other hematopoietic substances. Since the free folic acid level is very low in the blood of normal human beings receiving normal diets, this measurement alone would not be useful in assessing the nutritional state with respect to folic acid. Additional studies comparing the values obtained with different test organisms for determining folic acid will afford data on the nature of the compounds present in various tissues and body fluids.

#### SUMMARY

The level of folic acid in the blood of human subjects was determined with *S. faecalis* R and *L. casei* as the test organisms two, four, eight and twenty-four hours after the intravenous administration of 12 mg of pteroylglutamic acid or the equivalent of pteroyltri-glutamic acid. A prompt, rapid, and essentially equal rise in the blood levels occurred when these compounds were given. The level was maximum two hours after injection when the first analyses were made, was still elevated eight hours after injection, but was at a normal level twenty-four hours after injection.

The values obtained with *L casei* four hours after injection of the vitamin were somewhat higher than those obtained with *S faecalis* R when pteroyltri glutamic acid was administered indicating that some of the triglutamate was present in the blood for at least four to eight hours after injection. These data also show, however, that conjugases of the tissues were capable of a rapid and effective release of derivatives active for *S faecalis* R from pteroyltri glutamic acid.

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## STUDIES IN SERUM PROTEINS

### IV CLINICAL STUDIES EMPLOYING RAPID CHEMICAL FRACTIONATION PROCEDURES, WITH PARTICULAR REFERENCE TO THE FREQUENCY AND SIGNIFICANCE OF HYPOALBUMINEMIA

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**D**ETERMINATIONS of "albumin" by salt fractionation procedures have shown this fraction to be decreased in a number of clinical conditions.<sup>1</sup> However, the "albumin" values obtained by such methods are now known to include at least two of the fractions revealed by electrophoretic analysis, namely albumin and alpha globulin. For this reason, and because albumin and alpha globulin have been shown to bear an inverse relationship to each other,<sup>2</sup> a review of the frequency and magnitude of hypoalbuminemia in clinical conditions has appeared desirable.

Electrophoretic investigations have shown a significant decrease in albumin concentration in certain diseases,<sup>1</sup> but because of the limited number of samples which can be handled by an electrophoretic laboratory, the available data do not yet give a true picture of the frequency with which the various conditions responsible for hypoalbuminemia actually occur in clinical practice. Chemical fractionation methods have recently been shown to give results comparable to those obtained by electrophoresis<sup>2, 3</sup> and also to permit analysis of large numbers of samples on a routine basis.<sup>4</sup> The results to be discussed were obtained by the application of our chemical fractionation method to 500 routine sera.

#### METHODS

The general principles of the fractionation procedure used are as follows:

- 1 Total protein is estimated with the aid of Weichselbaum's biuret reagent.
- 2 A filtrate containing albumin plus alpha globulin is obtained by treating serum with sodium sulfate at a final concentration of 20.24 per cent. Protein in the filtrate is estimated by the biuret reaction.
- 3 A filtrate containing albumin alone is obtained by treating serum with sodium sulfite at a final concentration of 26.88 per cent. Protein in the filtrate is estimated by the biuret reaction.
- 4 The alpha globulin value is obtained by subtracting the albumin concentration from that of albumin plus alpha globulin.
- 5 Beta globulin plus gamma globulin is calculated by subtracting the albumin plus alpha globulin concentration from the total protein concentration.

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6 Gamma globulin is precipitated from serum by ammonium sulfate at a final concentration of 139M at pH 6.0 and controlled ionic strength. The precipitate is dissolved in dilute biuret reagent for the estimation.

7 Beta globulin is calculated by subtracting the concentration of gamma globulin from that of beta plus gamma globulin.

The beta plus gamma globulin component was not further fractionated in this group of patients except in the supplementary group of nephrotic patients presented in Tables IV and V. Complete globulin fractionations are now being performed on a second group of patients and will be reported later.

Detailed working instructions for the rapid estimation of total protein, albumin, total globulin, alpha globulin, beta globulin, and gamma globulin in 10 ml of serum will be published shortly.<sup>4</sup> The time required to carry through the entire procedure is only slightly more than twice that required for a determination of total protein and albumin by the Howe method.

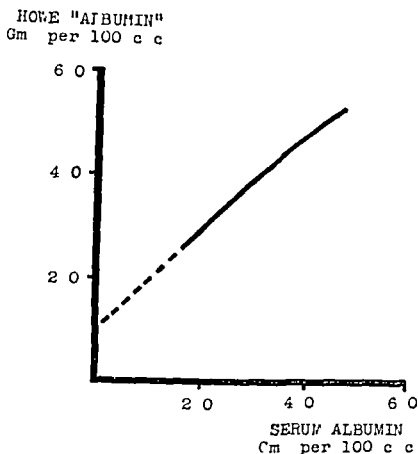


Fig. 1—Average relation between Howe albumin and serum albumin values in 100 unselected sera.

#### RESULTS

*Comparison of Howe "Albumin" Values With Albumin Values*—It is now generally recognized that the 'albumin' values obtained by the Howe sodium sulfate fractionation are always somewhat larger than the albumin values obtained by electrophoresis or by chemical fractionation techniques which have been adjusted to electrophoretic standards.<sup>2,9\*</sup> The difference between the Howe

\* For the purposes of clarity we have called the values obtained by the Howe procedure albumin, and those obtained by electrophoretic procedures or by chemical methods adjusted to electrophoretic standard albumin. This does not imply that the albumin fraction isolated by electrophoresis is necessarily a unitary fraction except in its behavior in an electrical field and under suitable precipitation conditions. There is in fact reason to believe that it may consist of at least two major subfractions and several other minor components.

"albumin" values and albumin values has been shown to be approximately equal to the alpha globulin concentration<sup>2, 7</sup> and to vary with the alpha globulin concentration<sup>2</sup>. In this series of 500 hospital patients, the average serum alpha globulin was 0.84 Gm per cent, with two-thirds of the values within the range of 0.6 to 1.1 Gm per cent. Most of the Howe "albumin" values were therefore from 0.6 to 1.1 Gm per cent greater than the albumin values determined simultaneously. Because this variation in alpha globulin values is not large in comparison with the size of the albumin fraction, the correlation between Howe "albumin" values and albumin values (Fig 1) is approximately linear.

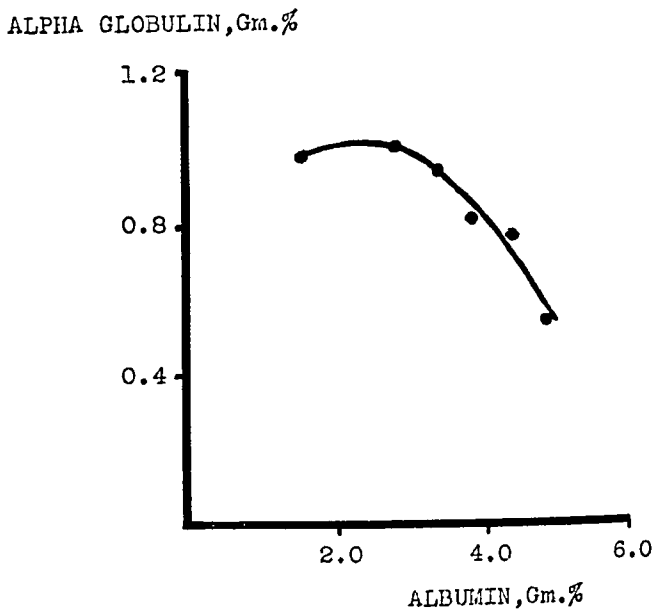


Fig 2—Average relation between albumin and alpha globulin in 500 unselected sera. (Each point represents the average of approximately eighty determinations)

Attempts to apply these data correlating Howe "albumin" values and albumin values to the derivation of a constant correction factor by which albumin may be calculated from Howe "albumin" values meet with certain definite objections, particularly when the accuracy of individual determinations is considered. Although the majority of alpha globulin values in our series fell in the middle range of 0.6 to 1.1 Gm per cent, eight patients had concentrations of 0.2 Gm per cent or less, and sixteen patients had concentrations of 1.5 Gm per cent or greater. Moreover, study of large numbers of samples has shown a definite tendency for alpha globulin concentrations to increase both absolutely and relatively as serum albumin concentrations decrease,<sup>11, 12, 13</sup> although the scatter of the data both in our series and in that of Chow<sup>11</sup> is very large.

Fig 2 shows the average correlation between serum albumin and alpha globulin values which obtains in our patients. At an average serum albumin of 4.81 Gm per cent, the alpha globulin concentration averaged 0.54 Gm per cent, and this value increased to an average of 1.00 Gm per cent at a serum



albumin concentration of 2.76 Gm per cent. Further decreases in albumin concentration did not lead to additional increases in the absolute values of alpha globulin, even though its relative concentration continued to increase. Because those circumstances which lead to a decrease in albumin values also usually lead to increased alpha globulin values, the net change in the "Howe albumin," which includes both albumin and alpha globulin, will tend to be less than the change in the albumin value.

A more serious defect of the Howe 'albumin' values may be deduced from Fig. 1, where the line which represents the relationship between Howe "albumin" values and true albumin values has been extrapolated to the base line. This projection, shown as an interrupted line, indicates that at an average true albumin concentration of 0.0 Gm per cent the Howe 'albumin' value will still be above 1.0 Gm per cent. It suggests that examination of a large series of samples should show the lowest Howe "albumin" values to be about 1.0 Gm per cent. This suggestion appears to have been borne out in our series of 500 determinations. Two patients showed Howe 'albumin' values between 0.9 and 1.0 Gm per cent, but all others had values of 1.30 Gm per cent or greater. This finding, when considered with the data on the frequency of hypoalbuminemia discussed below, indicates that Howe 'albumin' values conceal both the frequency and the magnitude of hypoalbuminemia.

*Findings in Hypoalbuminemia*—In this series hypoalbuminemia has been defined as a serum albumin concentration of 2.0 Gm per cent or less. Such values occurred in fifty samples from thirty-four patients, an incidence of 9.8 per cent. In twenty samples from ten patients (incidence 3.8 per cent) the albumin concentration was 1.0 Gm per cent or less. Howe 'albumin' values were below 2.0 Gm per cent in only ten patients and 1.0 Gm per cent or less in only two patients.

Electrophoretic studies have shown that marked hypoalbuminemia may occur in severe malnutrition, in the nephrotic syndrome, in chronic tuberculosis, with severe hepatic damage, during relapsing *Plasmodium malarie*,<sup>14,20</sup> and in the syndrome of idiopathic familial dysproteinemia.<sup>16,21,22\*</sup> Examples of each of these disorders, except for the last two, were found during this study, and a few additional diagnoses were noted.

Table I lists the diagnoses made in our cases of hypoalbuminemia and the frequency of each. Quantitatively the largest group was that of patients with the nephrotic syndrome. Two patients in this group had severe renal amyloidosis associated with long-standing tuberculosis, but the remainder of the patients

*Dysproteinemia* is a term which has been used to designate an abnormal distribution of the normal plasma protein components and is contrasted with *paraproteinemia* in which abnormal constituents are believed to appear in the plasma as in myelomatosis.<sup>6</sup> The syndrome of familial idiopathic dysproteinemia has been described by Homburger, Young, Petermann, and Relfenstein<sup>23</sup> as consisting of the familial occurrence of grossly abnormal plasma protein patterns not necessarily with manifest hypoproteinemia. Clinical signs included edema and peripheral circulatory abnormalities in affected women.

Armstrong<sup>24</sup> has referred to a group of similar cases as idiopathic hypoproteinemia and distinguishes two types: one in which the primary disturbance appears to be abnormal plasma protein formation, the other in which increased capillary permeability appears primary.

Wuhrmann and co-workers<sup>25</sup> also distinguish two types: one in which there is hypoproteinemia but with a normal electrophoretic distribution of components, the other in which the electrophoretic distribution resembles that of the nephrotic syndrome (nephrosis sine albuminuria). They have found that both types may be intermittent, edema appearing and diminishing with regular cyclic rises and falls in serum protein values.

were believed to suffer from chronic glomerulonephritis. No attempt was made to differentiate chronic from subacute glomerulonephritis. Three patients were diagnosed clinically as having hepatic cirrhosis. A third group of four patients was classified as having severe gastrointestinal involvement. This noncommittal designation was chosen because it was difficult to assess the relative importance of deficiencies in food intake or absorption and of functional hepatic impairment in the genesis of the hypoalbuminemia.

TABLE I CLINICAL DIAGNOSES IN THIRTY-FOUR PATIENTS PRESENTING SERUM ALBUMIN CONCENTRATIONS OF 2.0 GM PER CENT OR LESS

Nephrotic syndromes	
Nephrotic stage, chronic glomerulonephritis	18
Renal amyloidosis, secondary to tuberculosis	2
Severe hepatic dysfunction	
Hepatic cirrhosis	3
Severe gastrointestinal involvement	
Carcinoma of gastrointestinal tract	2
Intestinal obstruction	1
Following gastric resection	1
Disorders with reticulo-endothelial involvement	
Multiple myeloma	3
Hodgkins' disease	1
Dermatomyositis	1
Rheumatic fever	1
Idiopathic lymphatic atrophy with lymphopenia and hypogammaglobulinemia	1

A final group\* of patients were of interest because they presented a variety of conditions in which the reticulo-endothelial system was markedly involved and in which quantitative and qualitative alterations in serum globulins are ordinarily much more pronounced than hypoalbuminemia. This has been true in our study as well, most of our patients with multiple myeloma or rheumatic fever did not show a severe degree of hypoalbuminemia. Although at present the liver is believed to be the sole source of serum albumin,<sup>23, 24</sup> it is not yet established that the hypoalbuminemia seen in nephrotic patients with mild proteinuria, or in patients with reticulo-endothelial involvement, is to be taken as implying structural liver damage.

Table II lists the average values for the various serum protein fractions found in each of the groups mentioned and for the entire group of patients.

Hypoalbuminemia is most striking in the nephrotic syndrome (Table III). The average serum albumin concentration of the nephrotic patients was only 0.93 Gm per cent, while average serum albumin values exceeded 1.60 Gm per cent in all other groups. *Serum albumin concentrations of 1.0 Gm per cent or less were seen only in the nephrotic syndrome.* Although these concentrations appear small, our data show good agreement with the results of the few reported electrophoretic studies, which are summarized in Table IV.

The absolute values of the alpha globulin and the beta plus gamma globulin fractions of the nephrotic sera was only slightly larger than those of the average hospital patient. Possibly because of the tendency for gamma globulin to be lost in the urine with albumin in nephrosis, gamma globulin values in the

\*One patient who presented hypogammaglobulinemia, lymphopenia, lymphatic atrophy, and bronchiectasis will not be discussed in detail in this report. Complete data will be presented in a forthcoming case report.

TABLE II MEAN VALUES FOR SERUM PROTEIN FRACTIONS IN PATIENTS WITH HYPOALBUMINEMIA

DIAGNOSIS	NUMBER	TOTAL PROTEIN (GM %)	ALBU- MIN (GM %)	TOTAL GLOB- ULIN (GM %)	ALPHA GLOB- ULIN (GM %)	BETA PLUS GAMMA GLOB- ULIN (GM %)	A/G RATIO
Nephrotic syndrome	20	4.55	0.93	.62	1.15	2.53	0.25
Gastrointestinal involvement	4	3.35	1.74	.81	1.06	2.75	0.46
Reticulo endothelial system involvement							
Multiple myeloma	3	7.52	1.81	1.45	—	—	0.34
Other causes	3	6.60	1.63	1.97	0.80	4.10	0.33
Hepatic dysfunction	3	6.58	1.85	1.73	0.82	3.91	0.39
Hypogammaglobulinemia with lymphatic atrophy	1	3.50	2.00	1.50	0.90	0.60	1.33
Hospital patients (un selected)	400	6.40	3.21	3.11	0.84	2.27	1.06
W R H § pooled normal serum	†	7.01	3.11	3.4	1.10	2.14	1.16
Normal adults	‡	6.58	3.40	3.16	1.01	2.10	1.08

Total globulin values differ slightly from the sum of the globulin components because globulin fractionation was not performed in one or more patients.

† Average data from several determinations carried out on each of four samples of pooled normal human serum. Each pool contained sera from between 30 and 150 individuals. Chemical fractionation on two of the pools was checked by electrophoretic runs.

‡ Data from the Harvard Plasma Fractionation Program.  $\alpha_1$  and  $\alpha_2$  calculated for the omission of fibrinogen.

§ Michael Reece Hospital Serum Center.

patients may be markedly diminished and average gamma globulin values as low as 0.24 Gm per cent have been recorded in electrophoretic analyses<sup>16, 20, 22, 23</sup>. Gamma globulin levels in amyloidosis however tend to approach normal values,<sup>16, 20, 27</sup> and a tendency for gamma globulin values to rise during exacerbations of the nephrotic syndrome has been reported.<sup>20, 27</sup> The relatively normal values of serum gamma globulin in amyloid nephrosis may be associated with the presence of large amounts of gamma globulin in the urine.<sup>2</sup>

Chemical fractionation values on twenty samples from sixteen nephrotic children are presented in the lower portion of Table III. The average gamma globulin concentration is 0.30 Gm per cent a value in close agreement with the value of 0.33 Gm per cent obtained by averaging the reported electrophoretic data (Table IV). There is a striking correlation of albumin and gamma globulin values. Ten children who had serum albumin concentrations below 1.0 Gm per cent had an average gamma globulin concentration of 0.11 Gm per cent while six children with serum albumin concentrations above 1.0 Gm per cent had an average gamma globulin concentration of 0.62 Gm per cent. This result does not depend upon technical factors in the salting out method since we have shown that serum albumin concentrations do not affect the completeness of gamma globulin recovery by the method employed.<sup>4</sup> It would appear therefore that whether or not proteinuria is considered to be the sole cause of hypalbuminemia in nephrosis the same factors which are responsible for severe hypalbuminemia are also responsible for severe hypogammaglobulinemia.

As a consequence of the decreased gamma globulin concentrations the beta plus gamma globulin fraction which is roughly equivalent to the Howe globulin comes to consist almost entirely of beta globulin in the average nephrotic

patient (Tables III and IV) The serum beta globulin concentrations show an inverse relationship to the serum albumin concentration The ten children in Table III with serum albumin values below 1.0 Gm per cent had an average

TABLE III SERUM PROTEIN FRACTIONATION IN PATIENTS WITH THE NEPHROTIC SYNDROME  
(The samples in the lower portion of the table, for which complete fractionation data are given, were studied after conclusion of the series of 500 determinations with which the paper is chiefly concerned)

PATIENT	NUM BER OF SAM PLES	TOTAL PROTEIN (GM %)	ALBU MIN (GM %)	TOTAL GLOB ULIN (GM %)	ALPHA GLOB ULIN (GM %)	BETA PLUS GAMMA GLOB ULIN (GM %)	BETA GLOB ULIN (GM %)	GAMMA GLOB ULIN (GM %)	A/G RATIO
<i>Etiology Chronic Glomerulonephritis</i>									
A N S *	2	4.85	0.15	4.70	0.78	3.92	---	---	0.01
S P I *	2	4.70	0.15	3.95	1.55	2.40	---	---	0.03
B R O *	1	4.80	0.30	4.50	---	---	---	---	0.01
F E L *	5	3.62	0.38	3.24	1.10	2.14	---	---	0.12
K A P *	1	3.80	0.40	3.40	0.90	2.50	---	---	0.12
F O R *	2	3.60	0.40	3.20	0.50	2.70	---	---	0.13
B R Z	1	4.05	0.80	3.25	1.60	1.65	---	---	0.20
H O W *	1	5.20	0.80	4.40	1.00	3.40	---	---	0.18
N I S *	4	4.78	0.93	3.85	0.83	3.02	---	---	0.20
G U E *	1	5.00	1.00	4.00	---	---	---	---	0.20
G R A *	1	6.30	1.10	5.20	1.40	3.80	---	---	0.21
W I L *	1	4.40	1.10	3.30	---	---	---	---	0.33
D A H	1	4.10	1.10	3.00	1.00	2.00	---	---	0.37
S E D *	1	3.40	1.10	2.30	0.70	1.60	---	---	0.48
P A N	2	3.55	1.35	2.20	0.55	1.65	---	---	0.40
W Y Z	1	5.50	1.40	4.10	1.30	2.80	---	---	0.34
B O R *	1	4.30	1.40	2.90	1.20	1.70	---	---	0.48
E K T	1	4.00	1.80	2.20	0.90	1.30	---	---	0.82
<i>Etiology Amyloidosis During Chronic Tuberculosis</i>									
L U N	1	5.40	1.00	4.40	1.20	3.20	---	---	0.23
W A S	1	6.40	2.00	4.40	1.20	3.20	---	---	0.46
Average of 20 patients	31	4.55	0.93	3.62	1.15	2.53	---	---	0.20
<i>Etiology Chronic Glomerulonephritis (Complete Protein Fractionations)</i>									
S P I *	2	3.40	0.10	3.30	1.35	1.95	1.67	0.28	0.03
G R A *	1	3.20	0.20	3.00	1.10	1.90	1.80	0.10	0.07
C C C *	1	3.80	0.20	3.60	1.05	2.55	2.40	0.15	0.00
S E D *	1	4.40	0.20	4.20	1.70	2.50	2.35	0.15	0.00
Y O U *	1	4.30	0.30	4.00	1.70	2.30	2.21	0.09	0.00
A N S *	1	4.40	0.30	4.10	0.60	3.50	3.35	0.15	0.01
C C B *	1	3.30	0.40	2.90	0.85	2.05	1.90	0.15	0.14
F O R *	1	3.40	0.40	3.00	1.00	2.00	1.90	0.10	0.13
S I L *	2	4.60	0.70	3.90	1.35	2.55	2.17	0.38	0.18
H I G *	1	3.70	0.90	2.80	1.20	1.60	1.10	0.50	0.32
G U E *	2	5.15	1.00	4.15	1.35	2.80	1.40	1.40	0.24
C C A *	1	3.80	1.10	2.70	0.90	1.80	1.45	0.35	0.41
C C E *	1	3.70	1.30	2.40	0.90	1.50	0.80	0.70	0.21
L E O *	1	3.60	1.30	2.30	1.20	1.10	1.00	0.10	0.47
K V I *	1	4.70	1.50	3.20	0.90	2.30	1.80	0.50	0.41
W I L *	2	4.55	1.50	3.05	0.70	2.35	1.70	0.65	0.41
Average of 16 patients	20	4.00	0.69	3.31	1.12	2.19	1.89	0.30	0.21
Average normal values	†	6.58	3.42	3.16	1.06	2.10	1.32	0.78	1.19

\*Starred patients are children

†Data from the Harvard Plasma Fractionation Program<sup>22</sup> Table IV recalculated for the omission of fibrinogen

TABLE 11. REPORTED VALUES OF SERUM PROTEIN FRACTIONS IN NEPHROTIC SYNDROMES AS OBTAINED BY ELECTROPHORETIC AND BY CHEMICAL ANALYSIS

AUTHOR	NUMBER OF PATIENTS	TOTAL PROTEIN (GM %)	ALBU MIN (GM %)	TOTAL GLOB ULIN (GM %)	ALPHA GLOB ULIN (GM %)	BETA PLUS GAMMA GLOB ULIN (GM %)	BETA GLOB ULIN (GM %)	GAMMA GLOB ULIN (GM %)	A/G RATIO
<i>Etiology Glomerulonephritis</i>									
Electrophoretic fractionation									
Longworth and MacInnes	2 (2 adults)	4.87	0.71	4.16	2.14	2.02	1.74	0.28	0.17
Luetscher <sup>27</sup>	4 (3 adults, 1 child)	3.70	0.73	2.97	1.13	1.84	1.60	0.24	0.25
Florn and co workers <sup>20</sup>	6 (6 adults)	4.06	1.12	2.94	1.35	1.59	1.22	0.37	0.38
Wuhrmann and co workers <sup>16</sup>	4 (4 adults)	4.15	1.47	2.68	1.16	1.52	0.94	0.58	0.55
Malmros and Blix et	4 (4 adults)	5.44	1.52	3.92	1.60	2.32	1.26	1.06	0.39
Average electrophoretic data	20 (19 adults, 1 child)	4.44	1.11	3.33	1.47	1.86	1.36	0.51	0.33
Chemical fractionation (present study)									
Incomplete fractionations	18 (5 adults, 13 children)	4.24	1.29	2.95	1.07	1.88	--	--	0.44
Complete fractionations	16 (16 children)	4.52	0.71	3.81	1.00	2.82	--	--	0.19
		4.00	0.69	3.31	1.12	2.19	1.89	0.30	0.21
<i>Etiology Amyloidosis</i>									
Electrophoretic fractionation									
Malmros and Blix <sup>26</sup>	1† (1 adult)	4.90	0.70	4.20	2.40	1.80	0.90	0.90	0.17
Luetscher <sup>7</sup>	1‡ (1 adult)	3.59	1.31	2.28	0.83	1.45	0.51	0.94	0.57
Wuhrmann and co workers <sup>16</sup>	1   (1 adult)	5.20	1.90	3.30	1.36	1.94	1.34	0.60	0.58
Average electrophoretic data	3 (3 adults)	4.56	1.30	3.26	1.53	1.73	0.92	0.81	0.40
Chemical fractionation (present study)	2   (2 adults)	5.90	1.50	4.40	1.20	3.20	--	--	0.34

Total protein concentrations and schlieren patterns appear in the original article.<sup>20</sup> The relative concentrations of the various fractions were calculated by Luetscher<sup>7</sup> from the schlieren patterns. The absolute values quoted in the table were calculated from the data of these two sources with the omission of fibrinogen.

†The study of Malmros and Blix<sup>26</sup> was concerned only with patients with elevated sedimentation rates.

‡Electrolytic chronic osteomyelitis.

||Electrolytic not given.

|||Electrolytic chronic tuberculosis.

serum beta globulin of 2.09 Gm per cent, while those with serum albumin concentrations above 1.0 Gm per cent had an average serum beta globulin concentration of 1.36 Gm per cent. With the hyperbetaglobulinemia is associated the hyperlipemia of the typical nephrotic patient since the beta globulin components which are increased in nephrosis carry large proportions of protein bound lipid. The serum cholesterol values in such cases may be greatly elevated, they averaged 799 mg per cent in one series of six patients.<sup>30</sup> In such sera the presence of large amounts of lipid makes it difficult to determine the actual concentration of protein in the beta globulin fraction with electrophoretic methods since the lipids transported with the beta globulins increase the size of the beta globulin peak in the schlieren diagram, in this respect chemical fractionation is of distinct advantage, since protein alone is estimated.

Alpha globulin in the nephrotic patients exhibits neither the striking positive correlation with albumin values shown by gamma globulin nor the negative correlation shown by beta globulin. The group of children with albumin concentrations below 1.0 Gm per cent had an average alpha globulin of 1.19 Gm per cent, while those with higher albumin concentrations had an average alpha globulin concentration of 0.99 Gm per cent. These differences are probably not significant, and it seems likely that the failure of alpha globulin values to change significantly may well be due to the fact that its two major components have different physiologic activities. Alpha-1 globulin is a component which resembles albumin in its general behavior and renal clearance and may appear in large amounts in nephrotic urine.<sup>34</sup> Alpha-2 globulin, on the other hand more nearly resembles beta globulin in behavior and contains a certain amount of lipoprotein. A simultaneous decrease in the former and increase in the latter might well produce little net change in alpha globulin values.

It is difficult to compare the reported electrophoretic values in nephrotic patients (Table IV) directly with our values. Nineteen of the reported twenty electrophoretic values were obtained on adults while our values were obtained chiefly on children. However in the five adults included in our series of patients with nephritic nephrosis and in our two cases of amyloid nephrosis, the values obtained by chemical fractionation show less divergence from the mean electrophoretic values than do certain of the individual electrophoretic reports. From our data it appears likely that there may actually be some quantitative differences in the protein patterns of the adult and juvenile nephrotic patient. The average serum albumin is considerably higher in the adults, and from the preceding discussion one might expect this to be accompanied by beta globulin values which are not quite so high as in the juvenile nephrotic subject and by gamma globulins which are somewhat larger than in the younger group. On the whole, the review of electrophoretic data given in Table IV bears out these expectations and supports the view that the information gained by chemical fractionation is not essentially different from that obtained by electrophoretic analysis.

In the groups of patients other than the nephrotic subjects the average albumin values fell between 1.5 and 2.0 Gm per cent (Table II). In the patients with reticulo-endothelial involvement and with hepatic dysfunction, the total

serum globulin and the beta plus gamma globulin concentrations averaged more than 15 Gm per cent greater than those found in the average hospital patient. In the patients with gastrointestinal involvement and in the nephrotic patients total serum globulin and beta plus gamma globulin concentrations either were normal or were increased less than 0.75 Gm per cent.

## DISCUSSION

The application of chemical fractionation procedures to a large number of routine hospital determinations has confirmed in general the occurrence of hypoalbuminemia in those syndromes in which this finding previously had been reported by investigators who used electrophoretic analysis. In addition, hypoalbuminemia has been found in certain disorders which involve the reticulo-endothelial system and which ordinarily produce more pronounced alterations in serum globulins than in albumin. Statistically cases of the nephrotic syndrome accounted for over half of the patients in whom albumin concentrations of 2.0 Gm per cent or less were found and this syndrome accounted for all of the patients in whom albumin concentrations of 1.0 Gm per cent or less were found.

The finding that hypoalbuminemia is not only most frequent but also most severe in the nephrotic syndrome has certain implications for the pathogenesis of nephrotic edema. It is clear for example that whatever factors other than hypoalbuminemia may be involved<sup>17, 18, 30, 31</sup> hypoalbuminemia must play a larger role in the pathogenesis of nephrotic edema than in the pathogenesis of any other systemic edema. The low albumin concentrations, the suggestion that the serum albumin of the nephrotic patient may come to consist chiefly of an albumin subfraction of large molecular size<sup>16, 18, 31, 34</sup> and the absence of compensatory hyperglobulinemia appear satisfactory to account for the greatly diminished plasma oncotic pressure of the nephrotic patient.<sup>3, 35</sup> It seems possible that edema, in the nephrotic subject increases tissue turgor to a point where increased resistance to loss of fluid from the capillary balances to some extent the increased tendency to lose fluid which is the consequence of hypoalbuminemia. In this way, nephrotic edema becomes a dynamic factor which makes possible the maintenance of a more or less adequate circulating plasma volume. It is not clear why in the nephrotic patient dynamic compensation for

TABLE I. SERUM PROTEIN FRACTIONATION PATTERNS IN PATIENTS WITH HYPOALBUMINEMIA

FOUND IN	NEPHROTIC SYNDROME GASTROINTESTINAL INVOLVEMENT	HEPATIC DYSFUNCTION RETICULO-ENDOTHELIAL SYSTEM INVOLVEMENT	HYPOGAMMA GLOBULINEMIA WITH LYMPHATIC ATROPHY
Total protein	Usually low	Normal or high	Low
Albumin	In nephrotic patients may be below 1.0 Gm %	Above 1.0 Gm %	Above 1.0 Gm %
Total globulin	Normal or elevated less than 1.0 Gm %	Elevated more than 1.0 Gm %	Low
Alpha globulin	Normal or slightly elevated	Usually normal	Normal
Beta plus gamma globulin	Normal or elevated less than 1.0 Gm %	Elevated more than 1.0 Gm %	Very low
V/G ratio	Usually below 0.5	Usually below 0.5	High

decreased plasma oncotic pressure should depend largely upon alterations in interstitial pressure, while in most cases "compensatory" hyperglobulinemia (relative or absolute) aids in compensating for decreased oncotic pressure.

In patients with hypoalbuminemia, the presence or absence of "compensatory" hyperglobulinemia, the severity of the hypoalbuminemia, and the alterations in beta plus gamma globulin concentration make possible a rough differentiation of three patterns of hypoalbuminemia (Table V). In the nephrotic subject and in patients with gastrointestinal involvement, the pattern is that of hypoalbuminemia with little "compensatory" hyperglobulinemia. In patients with hepatic dysfunction and with reticulo-endothelial involvement, albumin concentrations are not so low as in nephrosis, and globulin concentrations are significantly elevated. The pathologic physiology of these latter two groups of diseases strongly suggests that the hyperglobulinemia is in fact not "compensatory" but an effect produced by involvement of the globulin-forming tissue in the disease process. It is possible that the differences in pathologic physiology which are reflected in the findings of Table V may, on occasion, be employed as an aid to differential diagnosis.

#### CONCLUSIONS

Rapid chemical fractionation procedures which give results approximating those of electrophoresis have been applied to a study of 500 sera received for routine analysis.

Systematic comparison of the albumin values obtained by this method with Howe "albumin" values shows the latter to conceal both the frequency and magnitude of hypoalbuminemia.

Serum albumin concentrations of 1.0 Gm per cent or less occurred only in ten patients with the nephrotic syndrome.

Serum albumin concentrations of 2.0 Gm per cent or less occurred in an additional ten nephrotic patients and in fourteen other patients who were grouped into the general categories: severe gastrointestinal involvement, hepatic dysfunction, and diseases with involvement of the reticulo-endothelial system.

The increase in serum beta globulin and decrease in serum gamma globulin which has been reported in nephrotic adults has been confirmed in a group of sixteen nephrotic children. There is a marked correlation between the degree of hypoalbuminemia, the magnitude of increase in serum beta globulin, and the magnitude of decrease in serum gamma globulin.

Increases in total globulin or in alpha globulin were slight or absent in the patients with nephrotic syndrome or gastrointestinal involvement. In patients with severe hepatic dysfunction or reticulo-endothelial involvement, total globulin and beta plus gamma globulin were increased, on the average, more than 1.5 Gm per cent.

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## FAILURE OF ANTIRETICULAR CYTOTOXIC SERUM IN ARTHRITIS

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THE imposing list of diseases claimed by Bogomolets<sup>1</sup> and his co-workers to have been benefited by the antireticular cytotoxic serum (ACS) includes also acute rheumatism. It is to be regretted that the translators were not aware that in part of the European literature this term indicates rheumatic fever. That the latter was meant by the Russian authors is evident by the qualification that antireticular cytotoxic serum is contraindicated in patients who suffer from endocarditis and myocarditis.

The sensational press notices omitted the word acute and both the public and physicians were fired with the expectation that a cure for all forms of rheumatism was discovered. A great clamor was raised and when the serum was produced in this country it was immediately introduced into the therapy of various forms of arthritis.

Previous studies of the reticuloendothelial elements in the synovial membrane and its role in the chemotherapy of rheumatoid arthritis<sup>2,4</sup> have made me aware of objections to the extended concept of the reticuloendothelial system (RES) and the therapeutic application proposed by Bogomolets and his school. However, for one engaged in the practice of rheumatology, an investigation of the effect of antireticular cytotoxic serum in rheumatic conditions became necessary in order to reach independent conclusions concerning its efficacy.

### PREPARATION AND DOSAGE OF ANTIRETICULAR CYTOTOXIC SERUM

The methods of preparation and standardization of antireticular cytotoxic serum were described by Marchuk<sup>5</sup> and modifications by American investigators<sup>6</sup> followed.

The majority of our patients received rabbit antireticular cytotoxic serum prepared in lyophilized form and supplied in a combination package. One vial contained 4 ml desiccated serum and another vial 4 ml of physiologic saline solution. In order to discover hypersensitivity, a preliminary intradermal test was carried out with normal rabbit serum. Some patients were given rabbit and/or goat antireticular cytotoxic serum.<sup>7</sup>

Two schedules were used. One followed the original directions of Bogomolets. A course consisted of an injection of 0.5 ml of antireticular cytotoxic serum followed after an interval of two or three days by 1 ml, and concluded with 15 ml after the same interval.

The second method of administration was developed by Straus and associates. Injections were given twice weekly starting with 0.2 ml of antireticular cytotoxic serum. The dosage was increased by 0.2 ml at each subsequent injection to a maximum of 2 or 2.5 milliliters. This dose was repeated up to six weeks depending on the response of the patient.

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I wish to express my appreciation to Wyeth Incorporated, Philadelphia, Pa. for the supply of ACS serum and extensive abstracts of Russian literature and to Dr. Reuben Straus for the supply of sera and treatment schedules.

†Produced by Dr. Straus in the Department of Pathology and Research, Cedars of Lebanon Hospital, Los Angeles, Calif.

With both methods the series can be given several times after an interval of at least four weeks. Before each series the intradermal test with rabbit serum was repeated in order to discover if hypersensitivity had developed in the interval.

In some of our patients we reduced the initial dose to 0.1 ml in order to avoid reactions. If there were marked reactions to rabbit serum, goat serum was substituted. If this also gave reactions, the treatment was discontinued. According to Bogomolets the use of antirreticular cytotoxic serum is contraindicated in acute and chronic endocarditis, myocarditis, nephrosis and nephritis, bronchial asthma, and exudative tuberculosis.

It is recommended that injections be given subcutaneously\*. The intravenous route was favored by some Russian authors. They (Kavetskiys) applied various tests to determine the functional state of the reticulo endothelial system and the effect of the antirreticular cytotoxic serum upon it. Most of these tests are too complicated for clinical use, none is specific. Only the sedimentation tests and the hemogram were carried out on our patients. A decrease in sedimentation rate and an increase in the percentage of monocytes and lymphocytes is supposed to indicate an enhanced function of the reticulo endothelial system.

Table I gives the results in sixty cases of the various types of rheumatic diseases.

TABLE I RESULTS OF ANTIRETICULAR CYTOTOXIC SERUM IN SIXTY CASES

TYPE	NUMBER OF CASES	IMPROVED		NOT IMPROVED	WORSE
		MODERATELY	SLIGHTLY		
Rheumatoid arthritis	30	1	4	16	9
Osteoarthritis	20	2	4	12	2
Fibrositis	10	2	1	6	1
Total	60	5	9	34	12
		(8.3%)	(15%)	(56.6%)	(20%)

#### RHEUMATOID ARTHRITIS

Thirty patients with rheumatoid arthritis received antirreticular cytotoxic serum. They represented a cross section of the adult form of the disease, as seen in our clinic and private practice. Numerous peripheral joints were affected. Cases of Strumpell-Marie spondylitis and Still's disease (adolescent rheumatoid arthritis) were not included. Twenty patients were women, ten were men. The ages ranged from 23 to 63 years. The average age was 45.3 years. The duration varied from two to twenty years, the average duration was five and one half years. The arthritis was mild in three patients, moderate in thirteen, and severe in fourteen. Twenty-three patients had received previously one or more courses of gold therapy and fourteen of these have had significant objective improvement or remission, lasting from one to five years.

Fourteen patients received one course of the short or long series of antirreticular cytotoxic serum. Sixteen patients were given from two to six series of injections. In the first group the course was not repeated because of local or generalized adverse reactions. During the period of antirreticular cytotoxic serum therapy, supporting nutritional, physical, corrective, and analgesic treatment was continued.

The results were evaluated after conclusion of the treatment and at a check up six months to a year later. One patient showed moderate and four slight subjective and objective improvement. The amelioration lasted not more than six weeks after completion of the treatment. Sixteen patients showed no im-

\*By Wyeth Incorporated and Dr. Straus

provement and nine became worse. The duration of the disease in the moderately improved patient was thirteen years and varied in the three slightly improved from nine to fifteen years. Among those not improved there were four cases of only two years' duration and four of less than four years' duration. Therefore responses were not better when the disease was of shorter duration or in a less advanced stage.

In a control group of 220 patients who were treated with various nonspecific measures alone, about 15 per cent had marked subjective and objective improvement. In 465 patients with rheumatoid arthritis treated by gold therapy, immediate marked objective improvement was achieved in over 52 per cent. Antireticular cytotoxic serum therefore was far less efficient than gold therapy and even nonspecific measures.

The following is evidence that the poor results with antireticular cytotoxic serum were not due to intractability of the material: twelve patients received gold therapy after antireticular cytotoxic serum had failed; of these seven had at least a moderate objective improvement. Four others were improved with other measures.

The initial sedimentation rate varied from 15 mm to 90 mm after an hour Westergren's method. There was no significant change in the rate after antireticular cytotoxic serum therapy even in the five instances associated with some degree of improvement. In patients who had severe reactions the sedimentation rate rose. No marked shift in the hemogram occurred. The number of injections and the length of treatment did not have a significant influence on the results. Three of the slightly improved patients received one to two series of three injections. On the other hand six of those who got worse received multiple courses with as many as fourteen injections per series.

#### OSTEOARTHRITIS

Twenty patients with osteoarthritis were treated. All had typical clinical and roentgenographic evidence of the affection. Fifteen had involvement of the spine either alone or associated with osteoarthritis of the hips, hands, and knees. Heberden's nodes were present in four persons. One patient had involvement of the shoulders. The ages ranged from 42 to 73 years; the average age was 55.8 years. Eighteen patients were women and two were men. This unusual distribution resulted from the prevalence of women among our patients. The duration of presenting symptoms was relatively short, from two weeks to three years. The average duration was one and a half years. The sedimentation rates were normal or only slightly elevated in several cases.

Under antireticular cytotoxic serum therapy only two patients were moderately improved and three slightly, twelve were unimproved and three got worse. One of the moderately improved patients relapsed one month after the treatment. Eleven patients unimproved by antireticular cytotoxic serum were given physical, nutritional, orthopedic, and climatic therapy. Of these three were unimproved, eight had significant improvement. Of those significantly

improved, six remained so at least for a year. These results clearly show antirreticular cytotoxic serum inferior to nonspecific measures commonly used in osteoarthritis.

#### FIBROSITIS

Ten patients were treated. The presenting symptoms were pain and stiffness of the various muscle groups, especially on arising and after remaining in one position. They were ameliorated after activity and warming up. The lower back, the shoulders, and the arms were involved. There was tenderness and spasm of the affected muscles. Fibrositic nodules could be palpated in some instances. The roentgenograms showed the joints to be normal. The sedimentation rates were normal or slightly increased. The ages varied from 32 to 50 years, with an average age of 43 years. The duration of the symptoms was from six weeks to five years, average duration, two years. Two patients were moderately improved, one was slightly improved, six were unimproved, and one got worse. One patient with fibrositis of the right forearm, which was moderately improved, had a severe local and systemic reaction, with fever, malaise, and headache. A week after the reaction had subsided the patient had a recurrence and spread of the symptoms to the left forearm and lower back. Granted that this group was more chronic and stubborn than the majority of cases, the results of the antirreticular cytotoxic serum in fibrositis were disappointing.

#### REACTIONS

Of the sixty patients, thirty-seven (62 per cent) had reactions, of these, fourteen (38 per cent) were mild, nine (24 per cent) were moderate, and fourteen (38 per cent) were severe. There was no significant difference in the degree of reactions in the various types of rheumatic diseases treated. 56 per cent of patients with rheumatoid arthritis, 70 per cent with osteoarthritis, and 60 per cent with fibrositis had reactions. Severe reactions occurred in 23 per cent with rheumatoid arthritis, 20 per cent with osteoarthritis, and 20 per cent with fibrositis. Mild reactions consisted of inflammation and tenderness of an area of several inches surrounding the point of inoculation. In moderate reactions the inflammation spread over a large part of the arm. In severe reactions it involved the whole arm to the elbow. The skin and subcutis became edematous but there was no suppuration. In one case of rheumatoid arthritis an effusion in the olecranon bursa occurred. The reactions started in six to twenty-four hours after injection. With the severe reactions there were low-grade fever of one to three days' duration, joint pain, malaise, headache, and lymphadenitis. In sixteen patients pruritus and urticaria occurred, in six, these reactions were general. Eczema under the axillae and thighs occurred in a patient with rheumatoid arthritis. In one patient with osteoarthritis, ecchymosis and purpuric spots developed around the puncture points and over the forearm. One patient had marked palpitation during treatment. Three osteoarthritic patients developed angioneurotic edema. In two patients the eyelids and in one the lips were involved for several days to two weeks.

The reactions were nonspecific manifestations of hypersensitivity to foreign proteins and/or allergy.

During and after the course of treatment a definite increase in hypersensitivity to antireticular cytotoxic serum occurred in some allergic patients. This is illustrated by a female patient with rheumatoid arthritis who developed urticaria in the first course after 15 milliliters. A second series was started after six weeks with only 0.1 ml and she developed a local reaction and urticaria after the second injection of only 0.2 milliliters. Previous to antireticular cytotoxic serum administration the patient had a severe urticaria after massive intramuscular doses of penicillin and beeswax in oil.

The majority of reactions occurred with increasing dosages of antireticular cytotoxic serum. When the Boomschtoets schedule of three injections was used the patients usually tolerated the first injection of 0.5 ml but reacted to the second (1 ml) or third (15 ml) injection. In the longer series starting with 0.2 ml most of the reactions occurred at the middle or end of the course when the dosage approached 1 or 2 milliliters. Two patients treated elsewhere with long series of injections manifested acute inflammation of the previously normal thumb and wrist joints. Prolonged series of injections with very short intervals were given for one or more years by some physicians. If antireticular cytotoxic serum should have a cumulative effect the prolonged therapy may act similarly to a blocking of the reticulo-endothelial system. In experimental animals Emerson, Ewing, and Thomas<sup>9</sup> produced severe macrocytic anemia by high dosage of antireticular cytotoxic serum.

#### DISCUSSION

Bach<sup>10</sup> in 1945 reported forty-eight patients with different types of arthritis treated by antireticular cytotoxic serum. Thirty-five of these had rheumatoid arthritis. Rogoff, Freyberg, Powell and Rice<sup>11</sup> in 1947 published results on the treatment with antireticular cytotoxic serum of twenty-nine patients with

TABLE II. RESULTS OF ANTIRETICULAR CYTOTOXIC SERUM IN RHEUMATOID ARTHRITIS

AUTHOR	YEAR	NUMBER OF PATIENTS	OBJECTIVE IMPROVEMENT		RELAPSED AFTER WEEKS TO 12 MO
			CASES	PER CENT	
Bach <sup>10</sup>	1945	35	3	9	-
Rogoff and co workers <sup>11</sup>	1947	29	3	10	-
Boots and co workers <sup>12</sup>	1947	34	7	20	2
Kling	1948	30	5	16	5
Total	1945-1948	128	18	14	9 (50%)

rheumatoid arthritis. Eight patients with rheumatoid spondylitis (Strumpell Marie type) were included in this series. Boots, Coss and Rigan<sup>12</sup> in 1947 analyzed results in thirty-four patients with rheumatoid arthritis. These authors concluded that the results of antireticular cytotoxic serum were discouraging or inconclusive.

Table II summarizes their combined results and my own in rheumatoid arthritis. Of 128 patients treated eighteen (14 per cent) had some degree of objective improvement. In the majority this improvement was only moderate or slight. In over 50 per cent relapses occurred after a short period. As

pointed out previously, an equal or better percentage of improvement was obtained by nonspecific measures

Moreover, Rogoff and co-workers<sup>11</sup> gave to a control group of fourteen patients with rheumatoid arthritis normal rabbit serum alone. Two patients (14 per cent) showed objective improvement and one had subjective improvement. The results equalled those obtained by antireticular cytotoxic serum. This suggests that any benefit may be due to a nonspecific serum effect rather than to the specific influence of antireticular cytotoxic serum. Bach used antireticular cytotoxic serum prepared in Bogomolets' Institute and sent to England. Stiaus<sup>13</sup> recently pointed to the possibility that this serum was inactivated in transit. He found a loss of antibody titer below significant levels in antireticular cytotoxic sera which he had received from Russia. However, this does not explain the equally poor results of the other authors mentioned who worked with antireticular cytotoxic serum produced in the United States and tested and approved in Dr. Stiaus' laboratory.

#### CRITICISM OF BOGOMOLETS' CONCEPT OF THE RETICULO-ENDOTHELIAL SYSTEM

Rheumatoid arthritis should be the most likely type of arthritis to respond favorably to antireticular cytotoxic serum, if Bogomolets' concept is true. Although its specific etiology is unknown, inflammatory reactions are present in the synovial tissues, in the subchondral bone marrow, in the lymph nodes, muscles, tendons, perivascular and perineural tissues, in the skin, and in other organs. Some investigators regard the disease as a systemic hypersensitive reaction, especially of the mesenchymal tissues, closely related to rheumatic fever. In the latter, according to Stiazhnesko,<sup>14</sup> antireticular cytotoxic serum is beneficial in the second, hypersensitive, stage. Therefore the failure of antireticular cytotoxic serum in rheumatoid arthritis is very disappointing. It was pointed out that Bogomolets and co-workers are not directly responsible for the use of the antireticular cytotoxic serum in these forms of chronic arthritis.

However, the greatly expanded concept of the reticulo-endothelial system, which, besides the macrophages and histiocytes in different organs and subcutaneous tissues, includes all unformed connective tissue and its derivatives, such as osteoid, cartilaginous, and synovial tissue, lends itself to the assumption that a general remedy has been discovered for treatment of every disease which is located in organs of mesodermal origin, for instance all forms of arthritis.

Against such unwarranted generalization it should be stressed that the function of the reticulo-endothelial system, which is established beyond a doubt as phagocytosis, storage and digestion of particulate living or dead matter such as bacteria, cells, fat, and colloidal dyes. The evidence of other important functions, such as antibody formation, is controversial.

MacMasters and Hudack<sup>15</sup> and Ehrlich and Harris<sup>16</sup> demonstrated by a series of ingenious experiments that the antibodies are elaborated within the lymphocytes and also possibly by the plasma cells. Under ordinary conditions these cells do not phagocytose or take up selectively vital dyes. The inclusion



of these cells in the reticulo endothelial system therefore is not justified Kass<sup>17</sup> proved that the lymphocytes produce the normal gamma globulins and the immune globulins Pomerat<sup>18</sup> has shown that the globulins are the effective fraction of antireticular cytotoxic serum The antigen is extracted from four parts of spleen and one part of bone marrow Therefore it contains a large portion of lymphocytic extract and the potency of antireticular cytotoxic serum as an antibody is at least partly due to the lymphocytic fraction Jaffe<sup>19</sup> rightly pointed out "It is not clear why the authors who have prepared or used extracts of the spleen identify the substances obtained from this organ with the reticulo endothelium since the spleen is composed not only of reticulo endothelial cells but also of other structures "

Applied to the rheumatic diseases, the objections can be summarized as follows

First it is not proved that the reticulo endothelial system is depressed in any type of these diseases

Second, the role of the reticulo endothelial system in pathogenesis and therapy has not been definitely established In the rheumatic diseases presented in this material and reviewed from the literature the results of antireticular cytotoxic serum have been discouraging

Third, it is doubtful that antireticular cytotoxic serum is a true antireticular serum

The consistent efforts of Bogomolets and his school have stimulated valuable research However, until accurate and practical methods of evaluation of the reticulo endothelial system and its role in a given disease have been elaborated and until a firm basis of action and efficiency of antireticular cytotoxic serum has been established and possible adverse effects have been eliminated, its introduction into general practice, especially in the therapy of rheumatic diseases, should not be encouraged

#### SUMMARY AND CONCLUSIONS

The therapeutic effects of antireticular cytotoxic serum were studied in sixty patients with different types of chronic rheumatic diseases On the basis of our material and a review of the literature it is concluded that there is no indication for antireticular cytotoxic serum in the treatment of osteoarthritis and fibrositis, which are ameliorated as a rule by physical, orthopedic, and drug therapy In rheumatoid arthritis and Strumpell Marie spondylitis which are so serious and often resistant to treatment, a trial of antireticular cytotoxic serum on the basis of an occasional significant improvement may be justified

Important objections to the concept of the extended reticulo endothelial system have been pointed out

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# THE EFFECT OF SUCCINATE IN MESCALINE HALLUCINATIONS

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## INTRODUCTION

INVESTIGATIONS by Quastel and Wheatley<sup>1, 2</sup> have indicated that the oxidation of succinate is not inhibited by the presence of barbiturates and certain of the narcotic amines as is that of glucose lactate and pyruvate. Following this very suggestive conclusion, Soskin and Taubenhaus<sup>3</sup> proposed the use of sodium succinate as an antidote to barbiturate poisoning, in which the succinate would serve as an oxidizable substrate until the barbiturate was eliminated from the body. Other investigators<sup>4, 5</sup> are divided in opinion as to the antidotal effect of succinate in barbiturate hypnosis. Comparative experiments by De Boer<sup>10</sup> concerning the diuretic effect of succinate and sucrose indicate that the decrease in sleeping time produced by succinate in barbiturate hypnosis may be correlated with the degree of diuresis produced.

Succinate also has been found to be of considerable experimental interest in the treatment of diabetic acidosis<sup>11</sup> in protection against poisoning by dithiols,<sup>12</sup> and in the place of salicylates in the treatment of rheumatic fever.<sup>13</sup>

In spite of this new interest, the possibility of using succinate in the treatment of narcotic amine depression has not been investigated. In particular we have become interested in the possible effects of succinate on the very striking visual hallucinations produced by mescaline (3,4,5 trimethoxy  $\beta$  phenyl ethyl amine). Mescaline effects have long held the interest of experimental psychiatrists and pharmacologists through the possible insights they may give into some aspects of the mechanism of hallucinosis. The literature on mescaline is vast with a particular emphasis upon visual effects. Kluver<sup>14</sup> in a comprehensive review outlines the visual effects characterizing the progressive intoxication of human subjects from a single dose of mescaline sulfate. These comprise a set of phenomena which are more or less constant for all individuals and include the development of (1) grating, lattice, fretwork, filigree, honeycomb or chessboard, (2) cobweb, (3) tunnel, funnel, alley, cone or vessel and (4) spiral.

Many other phenomena are on close examination nothing but modifications and transformations of these basic forms. The tendency toward geometrization, is expressed in these form constants is also apparent as the intoxication progresses. The forms are frequently repeated combined or elaborated into ornamental designs and mosaics of various kinds. The elements constituting these forms such as squares in a chessboard often have boundaries consisting of geometric figures.

These developments are accompanied by an equally vivid development of brilliant colors incorporated into the changing designs. The compounding of

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the designs leads to mental images of formed objects—faces, chairs, mountains, groups of people, and so on

In addition to the visual phenomena, certain physiologic effects will be mentioned later in the discussion which may have some bearing on the results of this study. The foregoing general outline regarding the development of visual effects is important for orientation to the chronological order and depth of the effects produced in the following experiments

#### EXPERIMENTAL

Experimental procedures were carried out in two directions (1) *in vitro* studies concerning the effect of mescaline sulfate on rat whole brain respiring on glucose, lactate, pyruvate, and succinate, and (2) *in vivo* studies on normal human subjects. The method followed in the *in vitro* studies was that of Quastel and Wheatley<sup>2</sup> and involved manometric measurements on chopped, mixed, whole rat brain tissue in Krebs-Ringer and (M/15) phosphate medium at a pH of 7.4. The mescaline was made up in Krebs-Ringer-phosphate media and neutralized to pH 7.4 before addition to the brain. The brain suspension (100 to 200 mg) was allowed to respire in air in the Warburg flasks in the presence of the drug until the uptake of oxygen had fallen to about 75 per cent of its

TABLE I

SUBSTRATE	OXYGEN UPTAKE* IN MM <sup>3</sup> AT 37° C FOR 0.5 GM RAT BRAIN FOR 2 HR		PER CENT DECREASE DUE TO MESCALINE
	WITHOUT MESCALINE	WITH MESCALINE (0.1%)	
Saline	300	290	3.0
Glucose 0.025%	620	390	59.0
Sodium lactate 0.025%	730	480	52.0
Sodium pyruvate 0.025%	790	450	53.0
Sodium succinate 0.025%	860	852	1.0

\*Each uptake expressed in the table is the average result of seven flasks

initial value, at which time the substrate was added. This initial period was usually about two and one-half to three hours. Readings were taken for two hours after substrate addition, and the per cent decrease in oxygen consumption by 0.5 Gm of rat brain due to exposure to mescaline at a final concentration of 0.1 per cent in the presence of glucose, lactate, pyruvate, and succinate was noted. These results are reported in Table I.

Subjects for the *in vivo* experiments were sophomore medical student volunteers. These individuals were given doses of 150 to 480 mg of mescaline sulfate intramuscularly and the hallucinations were allowed to develop until the designs observed were of great complexity and the colors of vivid and rapidly changing hues. Then sodium succinate (20 per cent in sterile water) was injected to the extent of 3 to 6 Gm intravenously, and the effects of the succinate on the hallucinations were recorded. The actual procedures were carried out in the Psychopathic Hospital, Department of Psychiatry, where all volunteers were

kept under careful observation at least twelve hours following the acute phases of the experiment

The results of these experiments are outlined in time sequence of the effects, the drug being administered intramuscularly (gluteally) in physiologic saline solution. The acute phases of the experiment were carried out in a dimly lighted quiet room with the subject reclining. Subjects were advised to keep their eyes shut and report frequently with prompting if necessary, the progress of the visions and any other symptoms involved during the course of the experiment. The experiments summarized in the following outline consist of (1) three trials illustrating individual sensitivity to mescaline throughout the acute course of mescaline intoxication (Experiments 1 through 3) and (2) four trials illustrating the effect of sodium succinate given intravenously near what appeared to be a peak effect of mescaline vision development (Experiments 4 through 7). Only those subjects showing a relatively high sensitivity as evidenced by profuse visions of great complexity and color were chosen for the succinate administration.

EXPERIMENT 1—20 year old male subject 132 pounds B P 134/74 pulse, 108/min

- 0 10 150 mg mescaline sulfate intramuscularly
- 0 55 First hallucinatory phenomena Black and red horizontal lines in definite sequence
- 2 58 Lattice work of snow crystals
- 3 00 Square building like objects in all colors in one plane
- 3 08 Swarms of little fish like objects
- 3 15 Perfectly drawn black circles on a yellow background
- 3 20 Purple snails with impression of the sky turned upside down
- 3 25 Looking down on a row of tents hung over a line with scraps of colored glass scattered about
- 3 30 Looking up at sky as through apex of a cone Bright waves of color
- 3 33 "All different colored stars"
- 3 35 Little doll like people coming out of a hall in every direction
- 3 40 "Different colored balls in tinsel going crisscross bumping and sticking together"
- 3 42 Increasing hesitancy to answer questions Fancy buildings lacework bricks "Just floating through"
- 3 45 One woman with a dog, man tips hat wind is blowing and woman is gone Smiles hesitates—"Everything tickles me"—"Most of the time I feel I'm just watching but once in a while I'm taking part"
- 4 00 Big smile Towers, not real towers lacey like Eiffel tower "When I see things, I want to keep them but can't"
- 4 10 "I feel big—big like a man with big hands that can break real big things" Negativism is extreme and subject answers questions with great hesitation.
- 4 30 Chicken coop with wire around—multicolored background "I see spots color spots" "You bother me" "I want to be left alone"
- 4 35 Things getting darker "I'm hungry"
- 4 40 Moved to convalescent ward and a meal
- 5 30 Still a few dull colors and simple designs
- 8 30 Almost all effects except a slight intoxicated feeling have passed off Pushing on eyeballs gives some bright colors
- 9 00 The next morning Feels fine Still some slight color intensification upon pushing on eyeballs

EXPERIMENT 2—23 year old male subject, 140 pounds, B P 119/29 pulse 90/min

- 10 0 210 mg mescaline sulfate intramuscularly
- 10 27 Fingers feel cold
- 10 8 A shiny peach colored ball in center of visual field

- 10 34 A lattice work of lines (when pressing on eyeballs)
- 10 45 Slight nausea and dizziness Sees nothing without pressing on eyeballs
- 11 00 Sees color background start to fill the field, but it passes away when he tries to observe it more closely
- 11 08 A dull green pattern which is greatly intensified by pressing on eyeballs
- 11 20 "Feel bulbous" Feels chair and arm to be out of proportion One side of body vastly larger than the other
- 11 30 Some hesitancy in answering questions with a tendency to gesture and shake head in answering rather than talk
- 12 00 No visual effects without pressing eyeballs
- 12 30 Feels room is distorted and hallway is funnel like but no color or design effects
- 2 00 Feels fairly normal

EXPERIMENT 3—25 year old male subject, B P, 128/68, pulse, 88/min

- 1 27 300 mg mescaline sulfate intramuscularly
- 1 40 Slight vertigo
- 1 50 Fullness in head with tingling in lips and around mouth
- 2 00 Legs feel unusually heavy Visual field somewhat "lighter" but no effects except a dull flicker when pressing on eyeballs
- 2 15 An additional 60 mg mescaline sulfate intramuscularly
- 2 40 No effect even when pressing eyes
- 2 50 Still no visual effects
- 4 20 Still no visual effects Pupils dilated somewhat

*Succinate Experiments—*

EXPERIMENT 4—Same subject as in Experiment 1

- 1 25 220 mg mescaline sulfate intramuscularly
- 1 50 Predominantly white and black line patterns over field changing to subdued green and blue on white Colors alternating quite rapidly
- 1 55 Lacework—white on black with color in the background getting progressively clearer
- 2 00 Small objects, like letters, revolving on lacework background, then little forms of bright red and orange making up a wheel
- 2 10 Many rings and various abstract patterns getting larger and brighter
- 2 30 Now many bright colored, multiple formed objects floating in air
- 2 45 Brilliant scenes of formed objects like arm chairs floating across valleys (5 Gm of sodium succinate injected intravenously, slowly, into arm vein)  
There is an almost immediate dulling of color and the formed objects are replaced by simpler patterns which are on a brownish background
- 2 55 A brownish haze with no patterns The subject is now fairly talkative and exclaims about the rapid change
- 3 30 The visual field seems lighter, but no designs or color even upon pressing on eyeballs
- 3 45 Pressing on eyeballs reveals some fretwork of dull brown
- 4 00 The fretwork design with some bright flashes seems more complicated but is clear only when pressing on eyes  
From this time on, light colors appear as background and develop with dull brown main of the lined fretwork type These effects gradually pass off by 9 00 P M

EXPERIMENT 5—26 year old male subject, 175 pounds, B P, 120/74, pulse, 90/min

- 1 25 480 mg mescaline sulfate intramuscularly
- 1 35 Six spike snowflakes clustered around a luminous ball of green yellow in the center
- 1 45 Bright yellow circle like a doughnut The background is marked off in horizontal lines
- 2 00 Feels "high," drunk Brilliant colors everywhere Feels very much disoriented in space
- 2 40 Complicated designs and rapidly changing colors The subject is very hesitant at this time in answering questions  
(6 Gm of sodium succinate injected intravenously)

- 0 50 "Color and design are gone except for dark blue black background  
 3 00 A few dark blue lines on dark background Intoxication is passing off The subject talks freely with no hesitancy Now feels somewhat euphoric  
 3 30 Still no color or design but feels a little euphoria again  
 4 00 Feels somewhat numb and euphoric with a light color tinged visual field with definite simple dull colored fretwork designs  
 7 00 Euphoria has passed off Very sleepy and exhausted

EXPERIMENT 6—22 year old male subject 131 pounds B P, 121/80 pulse 85/min

- 1 43 2.0 mg mescaline sulfate intramuscularly  
 1 50 Feels a slight 'heaviness' that comes and goes  
 2 30 Dull flashes of light with background of green fading to purple  
 2 45 A whirling design like a fan with violet spider webs  
 2 50 Colors brighter with much red and green and occasional designs in orange or yellow  
 2 55 People in silhouettes piling on top of each other on a background of Indian designs  
 A bright sun appears and spirals of bright yellow  
 3 10 Brilliant patterns with silhouette figures in all colors  
 (3.5 Gm of sodium succinate intravenously)  
 3 30 Colors have blurred and show mainly dull browns and greens on a black background  
 3 35 Visual field is still dull with simple fretwork design on dark background only upon pressing eyeballs  
 4 00 Feels fairly normal with color in background only on pressing  
 5 00 Color is returning with drunkenness and high feeling Colors quite vivid but patterns simple when pressing eyeballs  
 6 00 Still 'high' with some color mostly dull browns and greens or blues in background  
 Color brightens when pressing eyeballs  
 8 00 All color passed off with only some general visual field green or brown

EXPERIMENT 7—Same subject as in Experiment 5

- 1 45 450 mg mescaline sulfate intramuscularly  
 1 50 Gray snowflakes in black and white mosaic pattern  
 1 55 'Exhausted like at end of the day'  
 2 00 A little nausea  
 2 10 Black and gray white background with pin points of yellow and green Color fading  
 'in and out'  
 3 00 Design is increasing in complexity with light increase in color Fretworks cobweb designs 'melting in and out'  
 3 40 Progress in complexity with many details various parts of visual field show isolated  
 "independent" activity and development with effect of third dimension and perspective  
 3 00 A considerable increase in color with many formed objects—people animals in constant movement with a brick colored mosaic background  
 3 30 Much beautiful coloration and formed objects in three dimensions  
 (5 Gm of sodium succinate intravenously)  
 3 5 Faces of people and formed objects rapidly melt and dissolve with an almost immediate cessation of color effects  
 4 0 No patterns or color even when pushing on eyeballs Feels fairly normal with a little euphoria  
 4 00 Some vague design of a simple mosaic type when pushing on eyeballs  
 4 30 Still some simple design and a little color when pushing on eyeballs  
 5 30 Feels quite normal but sleepy and hungry  
 8 00 Sees vague gray on black, simple lattice work when pushing on eyes in a darkened room

*Miscellaneous Effects Observed in Some or All of the Subjects Which Are Caused by Mescaline and Which Are Antidoted Partially by Succinate*—Abolition of cremaster reflex, abolition of bladder filling sense, increased knee jerk reflex, depression and decrease in cooperation, with hesitancy of speech, space and time distortion, depression of respiration

*Effects Not Antidoted by the Succinate*—Dilatation of the pupil, hunger, tightness across the face

#### DISCUSSION OF RESULTS

The in vitro experiments carried out on rat brain illustrate and confirm in essence the information obtained by Quastel and Wheatley that oxidations by brain tissue are inhibited less by mescaline when respusing on succinate than when utilizing glucose, pyruvate, or lactate. It should be pointed out, however, that these inhibitions are obtained only if the brain is incubated with the drug in the absence of substrate for the initial two and one-half to three hour period. None or very little inhibition was obtained in this laboratory when the substrate was added at the beginning even in the presence of vastly larger quantities of mescaline (up to 0.5 per cent). In light of the fact that relatively small doses of mescaline, considering the total body weight of human subjects, produce profound effects within thirty to sixty minutes after the intramuscular injection, it is difficult to correlate the conclusions of the previous investigators regarding the in vitro effects of mescaline with the rapid, very dramatic effect which it produces in relatively small doses in vivo. Nevertheless, the implication of an antidotal effect by succinate, in greatly decreasing the complexity of designs and color, was strikingly demonstrated. The immediate effect of succinate seems to be the complete cessation of hallucinosis in some instances and in others a decided regression of the visions to an earlier, simpler design with great loss in color intensity.

That the effect of succinate in lessening the hallucinosis can be due to a diuretic effect seems also unlikely since the succinate effect is so immediate. In addition, after thirty to sixty minutes the succinate effect seems to lessen, and color and design again appear, though still much less profuse than before the succinate. This would seem to indicate the presence of still toxic amounts of mescaline, making an antidotal hypothesis based upon a diuretic action seem untenable. Possibly a considerable part of the injected succinate has been utilized by other cells of the body in thirty to sixty minutes.

Also observable was an antidotal effect of succinate with regard to the general demeanor and physiologic responses of the subjects. Thus the decrease in cooperation and hesitance in speech observed were replaced after succinate by a more normal attitude and readiness to talk. The bladder filling sense returned and there was some lessening of the knee jerk reflex toward the normal. Respiration was increased by succinate, as was observed by others.<sup>14, 15</sup> Regarding less of the mechanism, the effects seem most striking as described by one subject immediately after injection of succinate, "It was as if the colors and designs were being washed away."

#### SUMMARY

In vitro and in vivo experiments with mescaline sulfate were carried out. The in vitro experiments confirm the observations previously witnessed regard-



ing the lack of inhibitory action of mescaline on brain respiring on succinate and the definite inhibition of brain respiring on lactate, pyruvate, and glucose. In vivo experiments in human subjects demonstrate an antidotal effect of succinate on mescaline visions with regard to a decrease both in complexity of design and intensity of color. A consideration of the relatively large amounts of mescaline required to suppress brain respiration in vitro as compared with that required for the production of its in vivo effects, however, leaves some question as to the relation of the in vitro suppression to the process of hallucinosis.

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# ABSORPTION, DISTRIBUTION, AND RENAL EXCRETION OF MANDELAMINE (METHENAMINE MANDELATE)

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**M**ANDELAMINE, a urinary tract antiseptic now in clinical use, has been the subject of recent investigations in which it was reported<sup>1,2</sup> that Mandelamine, streptomycin, and sulfathiazole are approximately equal in their activity against organisms which commonly invade the urinary tract. It was found that organisms develop resistance to sulfathiazole and streptomycin rapidly, and to a remarkable degree, but, in contrast with these findings, resistance to Mandelamine did not appear at all. In order to gain further insight into the properties of Mandelamine, the following pharmacologic studies were undertaken.

## EXPERIMENTAL

*Chemical Methods*—Forty cubic centimeters of 0.1N sulfuric acid were added to solutions of Mandelamine (50 to 200 mg per cent) and the solutions were steam distilled at a rate to produce 40 cc of distillate in sixteen minutes. The distillate was analyzed for formaldehyde by means of Deninges' colorimetric method.<sup>3</sup> Two hours were required for full color development. Final readings, measured in a photoelectric colorimeter at 5,800  $\lambda$  units, were compared with a reference curve prepared by using standard solutions of formaldehyde. (One gram of formaldehyde is equivalent to 1.62 Gm of Mandelamine.) The sixteen minute distillation was selected because approximately 50 per cent of the Mandelamine is hydrolyzed to formaldehyde in this conveniently short period of time. Under these conditions analysis of Mandelamine solutions (3.12 to 200 mg per cent) gave an average recovery of 33 per cent (Table I). Results are, therefore, divided by 53.

The method is directly applicable to urine, but application to blood requires a protein free filtrate prepared according to the Haden modification of the Folin Wu procedure. Mandelamine added to whole blood in concentrations of 50 to 200 mg per cent is recovered as shown in Table II. The limit of sensitivity of the method, 0.6 mg per cent, corresponds to a concentration of 6 mg per cent in whole blood. In actual practice more reliable results were obtained when blood concentrations were 20 mg per cent or above.

Creatinine was determined according to the Folin Wu colorimetric procedure<sup>4</sup> modified for photoelectric measurement at 5,300  $\lambda$  units.

*Distribution of Mandelamine in Whole Blood*—Mandelamine (25 to 200 mg per cent) was added to heparinized dog blood and the mixture was shaken gently for thirty minutes. Samples of whole blood and plasma, analyzed for their Mandelamine content, gave the data (expressed in terms of blood and plasma water) shown in Table III. In order to approach physiologic conditions more adequately, the drug was injected intravenously into dogs, and samples of whole blood and plasma were then analyzed as before. The data indicate that the drug readily penetrates the red blood cell and distributes itself in proportion to the water content of whole blood. These experiments are of interest in con-

TABLE I RATE OF HYDROLYSIS OF MANDELAMINE

MANDELAMINE ADDED TO URINE (MG %)	PER CENT CONVERSION IN 16 MIN
3.12	54.4
12.5	53.6
50.0	51.3
200	52.9
Mean	53.0

TABLE II RECOVERY OF MANDELAMINE ADDED TO WHOLE BLOOD

MANDELAMINE ADDED (MG %)	MANDELAMINE RECOVERED (MG %)	PER CENT RECOVERY
200	201.3	100.7
198	210.5	106.3
198	210.5	106.3
198	208.1	105.1
198	208.1	105.1
95.2	90.4	95.0
95.2	90.4	95.0
49.8	50.8	102.0
49.8	50.2	100.8
49.8	49.4	97.2
50.0	50.7	101.4
Mean		101.4 ± 1.3

TABLE III PLASMA ERYTHROCYTE PARTITION OF MANDELAMINE (DOG)

PROCEDURE	AMOUNT ADDED OR INJECTED	MANDELAMINE RECOVERED (MG %)		P/B
		PLASMA / 0.92	WHOLE BLOOD / 0.79	
Mandelamine added to whole blood	24.8 mg %	29.6	28.6	1.05
	118.8 mg %	154.3	154.4	1.00
	118.8 mg %	132.6	139.2	0.95
	200.0 mg %	289.1	268.3	1.08
	200.0 mg %	220.6	217.7	1.01
	200.0 mg %	254.9	243.0	1.05
Mandelamine injected intravenously	750.0 mg /kg	80.2	78.9	1.02
	750.0 mg /kg	88.0	87.3	1.01
	750.0 mg /kg	13.7	71.3	1.03
	250.0 mg /kg	37.5	33.2	1.13
	250.0 mg /kg	59.1	26.8	1.09
	250.0 mg /kg	37.5	33.2	1.13
Mean				1.04 ± 0.05

nection with the following studies of the renal excretion of Mandelamine. It may be noted here that the erythrocyte membrane has been employed rather than the cellophane membrane frequently used in studies of protein binding.

*Renal Clearance and Distribution of Mandelamine in the Dog*—Four mongrel, male dogs (weighing 7 to 14 kilograms) maintained on a standard ration were fasted overnight before each experiment. At the outset the dogs were given 300 to 500 cc of water by stomach tube and light anesthesia was induced by intravenous injection of 60 per cent of the anesthetic dose of Nembutal. Creatinine (150 mg per kilogram) was injected subcutaneously and ten minutes later additional creatinine was given both subcutaneously (75 mg per kilogram) and intravenously (75 mg per kilogram). Mandelamine (250 to 750 mg per kilogram) was administered intravenously thirty minutes after the creatinine

TABLE IV RENAL CLARANCE AND DISTRIBUTION OF MANDELAMINE

DOG	WEIGHT (KG)	MANDOLAMINE INJECTED (MG)	TIME (MIN)	CUMULATIVE EXCRETION (MG)	PLASMA CONC/0.9% (G/G)	MANDEL AMINE CLEARANCE (G/G/MIN)	CREATININE CLEARANCE (G/G/MIN)	CLARANCE RATIO	VOI OF DIST.	PER CENT OF BODY WEIGHT			
1	116	10,200	16	35	172	-	23.5	-	5.9	43.4			
			31	326	105	19.8	27.9	70	9.4	69.1			
			46	767	96	33.0	48.1	69	9.8	72.1			
			60	1,201	89	37.3	49.5	75	10.1	74.3			
			74	1,655	84	41.5	55.3	75	10.2	75.0			
			89	1,854	76	18.7	40.6	43	11.0	80.9			
			104	2,286	71	43.8	44.3	99	11.1	81.6			
5	82	4,060	15	188	94	26.1	42.3	62	11.8	86.8			
			26	365	84	14.4	17.5	82	4.1	50.0			
			45	629	72	21.0	28.9	73	4.4	53.7			
			60	856	63	20.4	33.9	60	4.8	58.5			
			75	1,030	55	25.6	32.6	79	5.1	62.2			
			90	1,170	48	22.7	32.6	70	5.5	67.1			
			105	1,304	42	20.7	36.4	57	6.0	73.2			
4	109	7,500 (53% excreted)	14	254	99	22.8	32.8	70	8.0	80.5			
			29	636	90	28.5	33.7	85	7.1	86.6			
			43	997	84	19.7	40.8	48	7.3	67.0			
			50	1,201	76	30.2	44.8	67	7.6	69.7			
			74	1,619	70	32.8	46.9	70	7.7	70.6			
			89	1,873	65	25.9	44.2	59	8.2	75.2			
			104	2,175	59	33.5	41.5	81	8.4	77.1			
3	52	2,050 (15% excreted)	117	2,175	59	28.2	46.5	61	8.7	79.8			
			15	147	55	38.7	50.4	77	9.0	82.6			
			20	343	40	29.4	51.4	57	9.3	85.3			
			34	467	38	26.5	19.8	141	4.5	58.5			
			44	557	34	40.0	27.0	148	4.5	54.9			
			59	710	32	25.9	34.5	75	57.3	57.3			
			5	42	2,075 (69% excreted)	15	124	46	18.1	26.3	70	1.0	46.8
30	210	41				20.5	32.4	63	4.1	53.7			
35	480	35				28.2	33.2	85	1.8	53.7			
60	710	30				31.1	33.1	97	5.2	63.4			
* ANOVA F test statistic (D.F. 4, 30) = 0.67													
α = 0.05													
α = amount of drug in gms. 1 = amount of drug 1 cover 1 cumulatively in urine g. per gm													

Amount of drug excreted in urine (mg)     Amount of drug excreted in urine (mg)     Amount of drug excreted in urine (mg)

Under these conditions an adequate urine flow and a reasonably constant plasma level of creatinine were maintained. In the first three experiments eight urine samples were taken by catheter at measured intervals of approximately fifteen minutes each, and five samples of blood were collected during the test period. In the last two experiments four samples of urine and three samples of blood were collected. The plasma concentrations were plotted against time on semi-logarithmic paper and appropriate plasma concentrations were interpolated from the best straight line in accordance with the procedure described by Newman, Gilman and Philips. The data were used to calculate clearance ratios and volumes of distribution as shown in Table IV.

It is evident from the clearance ratios that Mandelamine undergoes reabsorption in the renal tubules. Closer scrutiny of the clearance data reveals two ratios which were manifestly out of line. These presumably resulted from analytic errors. It may be noted that the first Mandelamine and creatinine clearances were low. No explanation is offered for this observation but it is suggested that the depression in these clearance values may be the result of a temporary hypotension induced by the intravenous administration of the large doses of Mandelamine. No correction is included for possible binding of Mandelamine to plasma protein because the distribution of Mandelamine in whole blood indicates that the drug is freely diffusible. The volumes of distribution are consistent with the view that the drug is freely diffusible and rapidly attains equilibrium in its distribution in the compartments of body water.

The volumes of distribution observed within the first hour approximated the total volume of body water. With increasing time however the volumes of distribution increased to values as high as 87 per cent of the body weight. Because only 45 to 70 per cent of the total dose administered can be recovered in the urine, it is reasonable to assume that the apparent increase in the volume of distribution with time results from metabolism of the drug.

*Acute Toxicity of Mandelamine*—Jenkins, Jack and Drake<sup>6</sup>, Kolloff and Nelson and others have reported on the lack of toxicity of Mandelamine. In order to confirm and extend these findings the acute toxicity of Mandelamine was determined in mice (weight 17 to 22 grams, male, Carworth Farms (C.F.) No. 1 strain), guinea pigs (weight 300 to 400 grams, male), rats (weight 100 to 200 grams, C.F. male) and albino rabbits (weight 3 to 4 kilograms, male). Buffered solutions of Mandelamine were injected intravenously. In all species, signs of acute toxicity consisted of convulsions, extension of the extremities, respiratory paralysis, and cardiac arrest. The data based on ten to twenty animals per dose point are summarized in Table V. The notable lack of toxicity of Mandelamine is in agreement with work reported earlier.

TABLE V INTRAVENOUS TOXICITY OF MANDELAMINE

SPECIES	LD <sub>50</sub> (GM./KG.)	LIMITS OF ERROR (%)
Mouse	3.75	97-104
Guinea pig	1.50	89-112
Rat	>4.0	-
Rabbit	>2.0	-

TABLE VI CONCENTRATIONS OF MANDELAMINE IN HUMAN URINE

ORAL DOSE	SUBJECT	MANDELAMINE RECOVERED (MG %)																		
		TIME IN HOURS																		
		2	3	4	5	6	7	8	9	10	12	15	16	18	20	24				
1 Gm	1	50	346	777	142	983	833	736												
	(% excreted)	28	149	124	55	43	31	27												
	2	44	537	574	755	755	528	518		333	535									
	(% excreted)	06	39	69	62	49	60	83		52	64									
	3	128	658	692	114	794	123	823		335	503		149							
1 Gm repeated after 6 hr	4	40	177	344	-	107	958	707		-	469		261							
	5	82	191	29	103	91	891	726		595	278		29							
	6		225			194			190		153	726								
	7		106			919			246		170	112								
	8		51			706			346		106	803								
2 Gm repeated after 12 hr	9		745			852			658		92	745								
		57		542		159		310			217	325								
	11	73		217		248		157		118	65	287		434	248	-	207	147	96	

Buffered solutions of Mandelamine (1.25 to 10 per cent in physiological saline) were injected into the shaved abdominal skin of the rabbit. An irritant drug included to insure a positive response produced severe necrosis at the sites of injection, but the buffer saline and Mandelamine solutions gave uniformly negative results.

*Urinary Excretion of Mandelamine in Human Subjects*—Clinically 1 Gm. doses of Mandelamine are administered by mouth three or four times daily. One gram of Mandelamine therefore was administered orally to each of five male volunteers\* with the results shown in Table VI. It may be seen that effective concentrations<sup>1</sup> of the drug were attained in the urine within three or four hours, indicating a relatively slow absorption, and these concentrations persisted for at least another seven to eight hours. In a second experiment involving four normal male subjects 1 Gm. of the drug was given and after six hours a second dose was administered. Urine samples collected at the times indicated in Table VI were analyzed for their Mandelamine concentrations with the results shown. In a third experiment (undertaken because of the notable lack of toxicity of Mandelamine) twice the recommended dose, namely 2 Gm., was administered to two volunteers and the dose was repeated in twelve hours. As shown in Table VI this dose schedule also gave adequate urinary concentrations around the clock. It may be concluded from these data that 1 Gm. of Mandelamine administered by mouth three or four times daily will in the presence of normal renal function, produce and maintain antibacterial concentrations of Mandelamine in the urine.

TABLE VII. URINE CONCENTRATIONS AFTER 1 Gm. MANDELAMINE THREE TIMES DAILY FOR FOUR DAYS

SUBJECT	DAYS				MEAN VALUES
	1	2	3	4	
J. M.	130.7	101.0	65.5	110.4	102.9
J. F. R.	64.5	71.7	63.4	50.5	62.7
R. E.	93.9	52.2	73.6	75.5	73.3
M. R.	3.2	41.0	58.1	55.2	40.9
Z.	63.4	73.0	93.0	60.5	74.4
T.	15.5	106.7	77.4	101.0	103.4
G.	65.5	113.3	100.0	55.1	97.7
L.	100.9	105.5	104.5	100.9	110.7

In order to establish more convincingly the effectiveness of the accepted dosage nine volunteers were given 1 Gm. of Mandelamine by mouth at 9:00 A.M., 5:00 P.M. and before retiring. These experiments were continued for a period of four consecutive days. It should be emphasized that these subjects continued in their normal occupations with no limitations imposed upon the fluid or food intake. Daily urine samples collected at 10:00 A.M. were analyzed with the results shown in Table VII. All urinary concentrations were within or above the range of antibacterial effectiveness. The mean of all values was 133 = 308. Although the figures varied significantly among themselves it could be shown by analysis of variance that differences within individuals were no significant within the observation period of four days. It may be concluded

\*Commercial Mandelamine tablets were used in all human experiment.

therefore, that a dose of 1 Gm three times daily will, in the presence of normal renal function, produce antibacterial concentrations of Mandelamine in the urine

Carroll and Allen<sup>8</sup> have reported that, following ingestion of Mandelamine, the urine of 96 per cent of their patients became and remained acidic without other medication or restriction of diet or fluid intake. Our findings in normal subjects are in agreement with those of Carroll and Allen. Invariably the urinary pH was lowered, and in all instances a single dose of 1 Gm was sufficient to lower the urinary pH to about 6. The urinary pH was maintained between 5.8 and 6.4 when 1 Gm of the drug was administered three times daily for four days.

#### SUMMARY

Mandelamine readily penetrates the red blood cell and distributes itself in proportion to the water content of whole blood. In the dog the volume of distribution approximates the total volume of body water. The volume of distribution increases with time, presumably because of extrarenal activity. The drug undergoes tubular reabsorption in the dog. These observations are consonant with the view that the drug persists in the organism. In human subjects, 1 Gm of Mandelamine administered three or four times daily is adequate to produce and maintain antibacterial concentrations of the drug in the urine. The notable lack of toxicity of Mandelamine is confirmed.

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# LABORATORY METHODS

## IMBEDDING OF PATHOLOGIC SPECIMENS IN TRANSPARENT PLASTIC

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### INTRODUCTION

IN THE teaching of biologic sciences it is essential to be able to demonstrate specimens of animals and soft tissues. This is even more true of some of the medical sciences, particularly gross anatomy and pathology. The preservation of such specimens always has presented a very difficult problem. The most usual and probably the most satisfactory method at present in general use consists in fixing the organs or slices of organs in various fluids, most of which as a rule contain formalin, with the addition of various substances designed to retard as much as possible the loss of color and shrinkage. These specimens generally are mounted in round or square glass jars. Even with the greatest care, however, and with the best known procedures, this method offers numerous disadvantages. First of all, color preservation is good to fail for only a relatively short period of time, because on standing there is progressive bleaching. In the second place, shrinkage takes place with distortion of the specimen. Third, there are the factors of weight and difficulty in handling bulky museum jars with the danger of breakage, difficulty of transportation and so on. In addition, there is generally great difficulty in perfectly sealing such jars, with the necessity of adding or renewing the fluid from time to time, and finally, the preserving fluid becomes discolored and after a period of time needs renewing. Very often also turbidity occurs.

Employing substances generally known as plastics which can be transformed from the liquid to the solid state, successful attempts have been made particularly by Sando,<sup>1</sup> to imbed minerals, insects and similar structures in transparent blocks. However, the procedure involved the use of chemical dehydrating agents, and it could not be applied successfully to animals or organs of animals containing large amounts of water without considerable loss of form and color. The combination of drying from the frozen state (by sublimation of water vapor), impregnation of the dried tissue with the liquid monomer, polymerization and subsequent imbedding in a transparent solid plastic has yielded good preservation of both color and form of the most delicate human, animal and vegetable tissues.

The use of dehydration by sublimation of water from the frozen state was suggested by the fact that plasma and other complex colloidal substances can be dried without deterioration.<sup>2</sup>

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Very recently, Kampmeier and Haviland have published a method for the mounting of wet anatomic preparations in Castolite. This method does not preserve the natural color in specimens.

Thus far the work has been directed toward developing a satisfactory basic technique. Since the time of the publication of a preliminary paper<sup>4</sup> the method has been considerably improved and applied to imbedding of practically every organ of the human body and numerous other animal and vegetable specimens. The early promises of this method have been at least partly realized. The present publication deals mostly with presentation of the technique which has given the most constant and satisfactory results. This technique suggests itself for application for the collection of anatomic and pathologic specimens for teaching in schools, medical and veterinary, and for the teaching of biology, zoology, botany, and the allied sciences.

#### PROCEDURE

*Material*—The material used for imbedding is uninhibited monomer consisting of a mixture of 90 per cent methyl methacrylate and 10 per cent ethyl methacrylate. This mixture is generally packed in dry ice to avoid polymerization prior to use.\*

The uninhibited liquid monomer must be maintained in the refrigerator to avoid premature polymerization. Under these conditions the liquid monomer can be maintained for several months.

*Preparation of Specimens*—The technique described below is intended especially for slices of organs.

The fresh specimen should be trimmed carefully and freed as much as possible of excessive fat and loose blood or fluid exudates. Slices of organs need not be limited in width to any size but they should be reasonably flat and should not exceed 2 cm in thickness. Organs or slices of organs which have a tough capsule should be freed of it if possible. This is particularly true of the kidney.

*Freezing* Freezing should be accomplished in a relatively short period of time, but instantaneous freezing is by no means necessary or even desirable. It is important not to allow the specimen to remain exposed to the dry atmosphere of low temperature cabinets for too long a period of time, in order to avoid superficial drying which would cause loss of color and distortion in the finished specimen. The best technique for slices of organs is as follows. Into a pan of proper depth, preferably metallic (although glass will do almost as well), pour water to a depth of about 1 cm and allow it to freeze and cool to a temperature of about  $-20^{\circ}\text{C}$ . During this process a raised lump often forms in the middle. This should be carefully scraped off in order to have a perfectly flat surface. Immerse the specimen in water and while it is thoroughly wet lay it carefully on the formed base of ice. Allow it to freeze, so that it becomes firmly attached to the base of ice, then add in rapid succession thin layers of water (0.5 to 1 cm.) Each layer of water is allowed to freeze before addition of a new one. Continue the procedure until the whole specimen is covered with approximately 1 cm of ice (Fig 1). The block of ice is then removed from the form and trimmed so that there is no excessive ice on the sides. For whole organs, proceed as follows.

\*This material is commercially known as Plexiglas. It can be obtained from the Polymers and Haas Company, Philadelphia, Pa.

Prepare a base of ice as outlined. Place the organ, thoroughly wetted, on the base and allow it to freeze. When the specimen is thoroughly frozen and fixed to the base, with an atomizer spray it several times with a thin layer of ice. A layer of 1 or 2 mm in thickness is enough to protect the surface of the organ from drying and subsequent loss of color and shrinkage. The specimen is then completely imbedded in ice by addition of successive layers of water of about 1 cm in thickness. Carefully avoid pouring the water on the organ itself in order not to melt the glaze of ice.



Fig 1—The fresh specimen frozen and encased in ice

Before placing the frozen block in the drying apparatus it is desirable to chip off as much as possible the excess of ice in order to reduce the period of drying. Specimens may be preserved in the frozen state without appreciable deterioration for more than a year. This operation is not essential except as a timesaver. The ice blocks must be thoroughly chilled to  $-20^{\circ}\text{C}$  or less before they are transferred to the drying apparatus.

*Drying of Specimens From the Frozen State* Any apparatus of proper capacity designed for the drying of biologicals from the frozen state can be used for this purpose. It must be capable of maintaining a temperature of  $-12$  to  $-15^{\circ}\text{C}$  or less in the specimen itself while the water is being removed by

sublimation. An apparatus previously described<sup>3</sup> or any similar one has been found to yield excellent results. It is desirable to place the blocks of ice containing the specimens to be dried in a loosely tied bag made of a single layer of gauze (12 mesh). The wrapped specimens are placed in wire baskets or on supports of the same material and so arranged as to allow a free flow of the water-vapor. For medium sized specimens (approximately 5 to 7 cm in over all thickness and of any width fitting the drying chamber) and with the apparatus mentioned, five days are sufficient for complete drying. The water jacket heating the drying chamber should be maintained at a temperature of 37 to 40° C. Higher temperatures may be injurious to specimens containing large quantities of fat. Very large organs such as an entire brain require about ten days for complete drying. Certain specimens, for example an entire animal or fetus, containing trapped air, such as that contained in the gastrointestinal tract, or soft tissues in a hard or bony shell at times show some distortion from shrinkage in the final specimen. This can be avoided by drilling holes before freezing. Otherwise drying from the frozen state, when properly carried out, causes no distortion of the specimen nor loss of color or structure.

*Trimming and Preservation of Dried Specimen.* Once the specimen is dried it should be carefully maintained so by placing it in a glass desiccator containing a suitable dehydrating agent, such as magnesium perchlorate (trihydrate). Specimens may be kept in the dried state before imbedding for several weeks or even months without appreciable deterioration. For optimal results, however, the dried specimen should be imbedded as soon as convenient. The specimen can be trimmed with a very sharp knife in order to remove all loose portions and for the purpose of improving the specimen. It has been noted that retrimming of a slice of organ after drying for the purpose of obtaining a very smooth and even surface allows a much better view of the intimate structure of the tissue. Excessive fat can be removed readily at this time, avoiding damage to the specimen. Dust and loose particles must be removed carefully from the surface of the organ with a soft brush.

*Saturation With the Liquid Monomer.* The dried specimens appear to have lost their color and texture. These are restored immediately upon immersion in the liquid monomer at room temperature. It is most essential to obtain a very thorough saturation of the specimen with the liquid monomer, which entirely replaces all spaces previously occupied by water. This is accomplished by submitting the jar containing the specimen to a high vacuum in a protected desiccator. As a result of the procedure of producing a vacuum and releasing it many times in succession, it will be noted that the specimen will sink in the medium and will issue no more bubbles. At this stage the specimen is ready for the passage to the thickened monomer, unless it is desirable to prolong the immersion in the monomer for the purpose of removing excessive amounts of pigment, a procedure which should be applied to such organs as the liver. With specimens of liver or organs containing other diffusible pigments or fat, the liquid monomer should be changed a number of times during the process of saturation, until it remains clear and practically colorless.

*Preliminary Partial Polymerization of the Medium —**Equipment and Reagents*

1,000 c c Erlenmeyer or Florence flask (Pyrex)

500 c c of the mixed uninhibited monomer

100 mg benzoyl peroxide

Water bath, electrically heated and with spark proof switch

A chemical hood If provided with a motor driven aspirator the motor should be of the induction type (spark proof)

*Procedure* To 500 c c of the monomer in the Erlenmeyer flask add 100 mg of benzoyl peroxide Immerse the flask so that the level of the monomer is at the level of the water of the water bath maintained under a hood Bring the water to the boiling point The boiling point of the ethyl methacrylate is about 110 C, but the polymerization is an intensely exothermic reaction and the monomer will soon boil Remove the flask from the water bath the monomer will boil without additional heat Allow boiling to proceed for two to three minutes Carefully cool without unnecessary jarring but with a constant swirling motion under running cold water until bubbling ceases Continue cooling for approximately ten minutes until a temperature of about 40 to 45 C is reached Place in the refrigerator (4° C) until ready to use The material can be thus stored for several weeks

Since the vapors of methyl methacrylate are highly inflammable they should be kept from all open flames sparking switches motors and so forth If possible, place switches outside the hood At all stages the prepolymerization process should be closely observed since the reaction is intensely exothermic and the monomer will solidify or boil over and form an opaque mass

Amounts larger than 500 c c may be prepared However greater difficulty is encountered in cooling rapidly and therefore the monomer may go on to complete solidification

The consistency of the final product is directly proportional to the length of time the monomer is allowed to boil The times given here produce material which is about the consistency of extra heavy oil or molasses

*Imbedding of Specimens* —For the imbedding of specimens and the preparation of solid bases a temperature of 40 to 45 C is generally employed For this purpose any hot air oven may be used provided the thermoregulator is spark proof A thermoregulator of the sealed mercury type is satisfactory

When large specimens are imbedded the heat of polymerization may be sufficient to produce a temperature considerably higher than 40 to 45 C, and if the excessive heat is not rapidly dissipated bubbling will occur and thus spoil the specimen In these instances a proper temperature can be maintained by a blower, activated by a thermostat set to close the motor circuit at 46 C (Fig 2)

It is desirable to use glass containers for the final imbedding Metallic containers have not proved as satisfactory Ordinary square refrigerator storage containers or baking dishes as well as glass photographic trays have been used with good results It is preferable to prepare the receptacles with a hard base

of plastic on which to rest the specimen. This hard base is prepared by pouring into a clean dish of suitable size and depth a layer of about 2 cm of the partly polymerized monomer. For this purpose very thick medium can be used. Cover the dish with two or three layers of cellophane, tightly fitted, and place in the refrigerator until the medium is absolutely free of air bubbles. Place at  $45^{\circ}\text{C}$  until the medium is entirely hard. The base is now ready to receive the specimen. The following procedure is recommended.

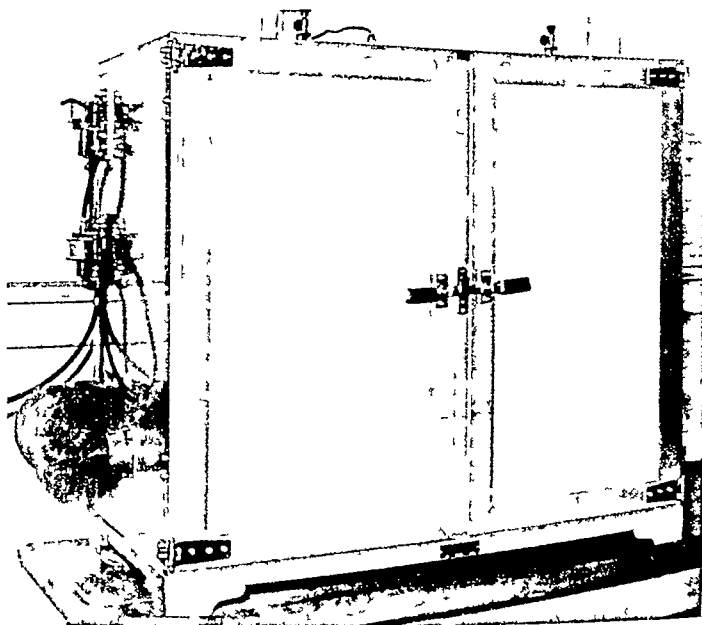


Fig. 2—Oven with blower

For flat slices of organs, or thin organs such as intestines, pour over the preformed hard base a small amount of the liquid monomer and with it thoroughly wet the base as well as the sides of the container. Pour the liquid off and drain rapidly. Pour over the hard base a layer of the thickened monomer calculated to be just enough to cover the specimen. If bubbles have formed, cover with cellophane and place in the refrigerator until they all have disappeared. Do not allow the poured thickened medium to stand for any period of time without a cover, particularly at room temperature or in the oven, because a tough film forms which renders subsequent operations difficult. When the medium is freed of air bubbles, remove the specimen from the monomeric medium and place it edgewise in the thickened medium by lowering it very gradually in such a manner as to avoid trapping air bubbles. It may be necessary at this time to add some of the thickened medium to cover the specimen completely, but under no condition add at this time a layer thicker than 2 to 3 centimeters. Cover the container with cellophane, and if air bubbles have been formed, replace in the refrigerator until clear. Then set in the oven at  $45^{\circ}\text{C}$  for polymerization. A block 3 cm thick will harden in approximately one week.

at 45° C. Successive layers of 1 to 2 cm. in thickness may be added to obtain a block of suitable size.

*Imbedding of Large Irregular Organs* The technique just described applies well to slices 1 to 2 cm. thick of any organs such as liver, spleen, lymph nodes, thyroid, lungs, kidneys, tumors, and to flattened out specimens from large vessels, intestine, gall bladder, and so on.

Large irregular organs, however, such as heart, lung, uterus, brain, and whole liver, require considerably more time and care. It is generally necessary

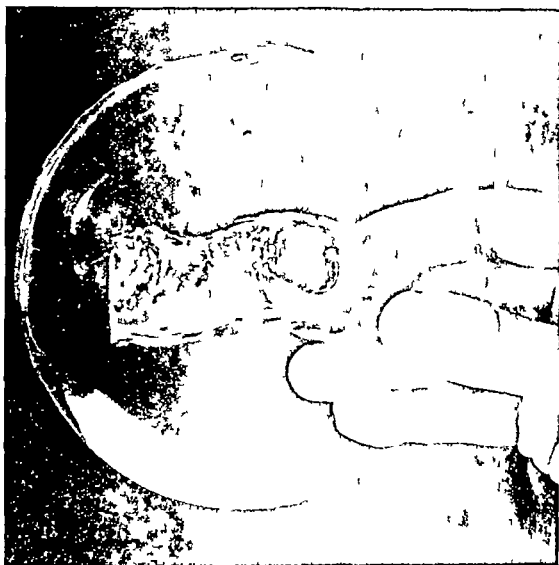


Fig. 3—Specimen imbedded in the block of transparent plastic

to begin the operation by preparing a base of suitable size. When this is obtained, the organ which is removed from the liquid monomer is coated with a thin layer of thickened monomer by immersion and placed on the base. The container with the base and the specimen is then very carefully sealed with many layers of cellophane or similar material. It is placed in the refrigerator so that it is thoroughly freed of bubbles and then placed in the oven at 45° C. This process is repeated until the organ is covered with a substantial layer. Great care must be exercised to cover the whole organ and to avoid trapping air bubbles. When the organ is thus glazed and fixed to the base, proceed carefully to imbed it by adding successive layers not exceeding 5 cm. in thickness in order

not to soften excessively the glazing on the organ. Whereas the imbedding of thin slices of organs offers no difficulty, the imbedding of entire large specimens challenges the skill and ingenuity of the operator. The results however are well worth the effort.

*Finishing of the Specimen* When the specimen is completely solidified, the edges of the plastic are detached from the glass with a sharp knife. On cooling, the blocks detach themselves from the walls of the container and will easily fall out with gentle tapping (Fig. 3). The finishing of the block is carried out as follows. The solid block first is rough-cut to the approximate size and shape of the final block desired with an ordinary band, circular, or jig saw. If a lathe is available it is well to turn the faces of the block down, since this will insure an absolutely flat surface. Since Plexiglas has machining qualities similar to those of brass and copper, metal cutting tools of this type can be used. A coolant (water, or soap and water) may be used if desired. If no lathe is available the block may be faced to a smooth (not accurately flat) surface by fastening emery paper to a block of wood and hand sanding with a circular motion. It is advisable to start the sanding with a coarse grade of emery paper and finish with a fine grade. If a sander is available the work may be accomplished much more quickly, but the final sanding should be a wet one, which will give a soft satin finish that can be buffed easily. Best buffing results are obtained with a very soft, open type of buffing wheel and an abrasive which is a combination of fine alumina with wax or grease binder, and a polishing tallow. The block, when finished, should have a high luster and be free from color and as transparent as the finest optical glass.

*Reimbedding After Cutting of Imbedded Specimens* To obtain a perfectly smooth surface of slices of organs or tumors, the imbedded specimen can be cut through with a fine band saw or similar tool and then polished as outlined to the point of a smooth, even, perfectly flat surface, or most of the clear block can be cut away and the specimen made flat by dry grinding. The specimen is then reimbedded as follows. Place the entire block with the exposed tissue surface uppermost in liquid monomer, and by means of high vacuum resaturate the tissue. Place face up in a suitable container and proceed to reimbed, following exactly the technique outlined previously. To surely avoid formation of bubbles on the raw surface of the exposed organ, it is desirable to store the specimen overnight in the icebox prior to polymerizing at 45° C.

Very thin, practically transparent sections can be obtained by proceeding as outlined and then cutting, polishing, and imbedding the other surface of the slice. These specimens are particularly valuable for teaching.

*Removing Trapped Air Bubbles* Trapped air bubbles can be removed from an otherwise satisfactory block by cutting and/or drilling with an instrument actuated by a high-speed motor, 5,000 revolutions per minute are satisfactory for this purpose. A tool with a flexible shaft is most desirable. Several types of drills may be obtained for practically any purpose.



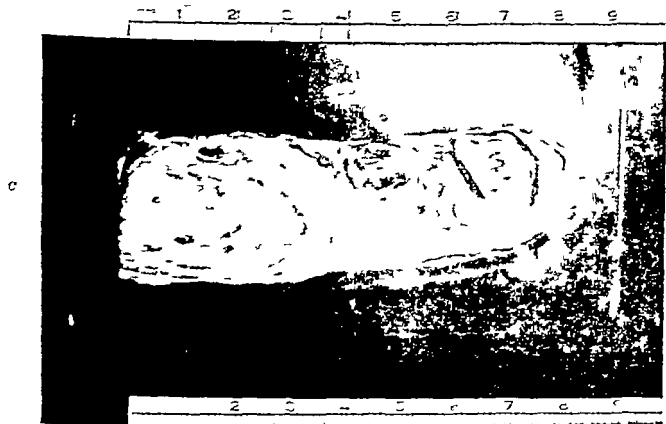
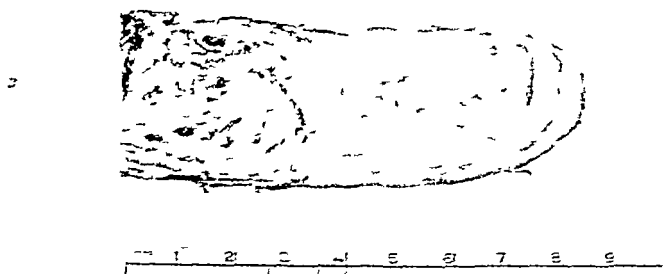
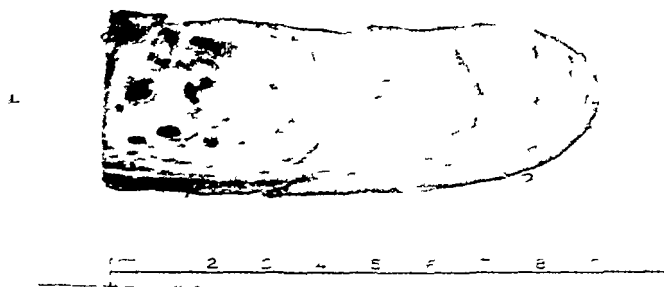


Fig. 1. A. Fragment of tissue of the liver. B. Section of the liver. C. Section of the liver. (The scale bar is in centimeters.)

*Commonest Failures in Imbedding of Specimens in Plexiglas*—These can be listed as follows

- (1) Loss of color Cause Previous fixation in formalin or prolonged immersion in liquid monomer
- (2) Shrunken portions Cause Incomplete immersion in water during freezing
- (3) Turbidity of the medium Cause Improper or incomplete drying, or excess of fat in the specimen
- (4) Formation of bubbles Cause Excessive heat during polymerization
- (5) Diffusion of pigment from the specimen Cause Too slow polymerization A common occurrence with certain specimens, such as liver
- (6) Disintegration of the specimen Cause Generally too prolonged exposure to monomer at high temperature
- (7) Fuzzing of the specimen at the periphery Cause Unknown Specimens thus affected may be cut so as to expose the inner portion and reembedded with good results
- (8) Formation of white precipitates Cause Unknown A not uncommon occurrence in imbedding very large specimens such as hearts and lungs Avoid prolonged immersion in partially polymerized methyl methacrylate by adding thin successive layers
- (9) Brittleness and loss of structure in cutting Cause Improper impregnation of dried specimen with liquid monomer

#### DISCUSSION

With the process described one can obtain preservation of the color and form of human specimens and similar material not possible with any other method known to us. These points are illustrated in Figs 4 and 5. Fig 4, A shows a fresh slice through a portion of a large xanthoma of the knee, with areas of hemorrhagic degeneration. Fig 4, B is the same specimen after drying and trimming. Fig 4, C shows the specimen imbedded in the finished block. Figs 1 and 3 show the same specimen frozen in a block of ice and at the stage of imbedding in the rough block of Plexiglas. Fig 5 shows the tumor preserved in a block of Plexiglas and a similar portion preserved in Klotz solution for a period of one year. Fading is already very noticeable in the specimen preserved in fluid.

For a slice of any organ up to 1 or 2 cm in thickness, it is approximately three weeks from the time the specimen is frozen to the time the finished block is obtained. The technique of cutting imbedded specimens, polishing the cut surface, and reembedding is recommended for optimal results. This process causes thorough impregnation of a perfectly smooth section of the specimen, particularly in such organs as liver, kidney, lung, and so on, thus permitting observations of minute details of the structure.

The technique for the imbedding of sections of organs, of tumors, or of entire animals can be said to be fairly well standardized and to produce fairly uniform and satisfactory results. Less constant results are obtained in im-



Fig 5 —Left portion of a xanthoma of the knee preserved in Klotz solution for a period of one year  
Right similar portion of the same tumor preserved in a block of Plexiglas



bedding entire large organs, such as an adult heart, lung or brain. With this type of material the results have been at times brilliant but not uniformly so, and failures have been just as common as successes. It is expected that improvement in the technique of imbedding and possibly in the preparation of the monomer employed may greatly aid the solution of the problem. With proper illumination, transparent blocks of Plexiglas containing organs or portions of organs have a particularly lifelike aspect. These specimens can be handled readily without danger of breakage and they remain absolutely unchanged for periods in excess of at least five years.

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# A CONVENIENT AND RAPID PROCEDURE FOR TOTAL CHOLESTEROL ESTIMATION USING AN ACID CHLOROFORM EXTRACTION

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THIS paper describes a simple rapid method for the determination of total cholesterol in blood serum.

The alcohol-ether extraction methods are time consuming because they require transfer and evaporation of the solvent<sup>1-4</sup>. Recently methods have been reported for the determination of total cholesterol in one test tube by the addition of acetic anhydride directly to the blood serum in the presence of sodium sulfate<sup>5</sup> or of dioxane<sup>6</sup>. These methods require procedures which need several hours for completion.

The method described herein will yield the desired result in approximately forty-five minutes. It lends itself to use in the routine laboratory in which large numbers of determinations must be done simultaneously. For example, thirty total cholesterol determinations in duplicate may be completed in two hours.

The present procedure depends upon the fact that dilute acids or alkalis, or saturated solutions of sodium sulfate, will split cholesterol from proteins in the presence of chloroform. Simultaneously, when this extraction is carried out in a shaking machine, the chloroform completely extracts the cholesterol.

The preferred procedure, therefore, consists of placing the serum in a test tube, adding dilute sulfuric acid and chloroform, stoppering the tube with a fat free rubber stopper, and centrifuging. The precipitated protein collects at the interface of the acid and the chloroform. The supernatant liquid and the protein button may be aspirated off with the aid of water suction and an aliquot of the chloroform layer taken. Acetic anhydride is added and the color is developed at constant time and temperature in the dark by the addition of concentrated sulfuric acid. This color may now be read in a photoelectric colorimeter.

When this extraction procedure is used, maximum color development occurs in seven minutes at 25 to 26° C. The color is therefore developed in a constant temperature bath at 25 to 26° C. and read after seven minutes.

Fig. 1 is a straight curve obtained by plotting concentration against the reading in a Klett-Summerson photoelectric colorimeter with a No. 60 filter. A similar curve is obtained using 625 m $\mu$  as the absorption beam with the Coleman spectrophotometer.

A box was designed to shake simultaneously 150 to 200 tubes for cholesterol extraction. This box is mounted on a standard large sized Kahn reciprocating shaker. Fig. 2 shows this box, made of five-ply wood, the partitions, and the rubber-faced plungers. This shaking box is also suitable for holding four bottles in place and shaking them simultaneously.

From the Biochemistry Laboratory, Jewish Hospital of Brooklyn.  
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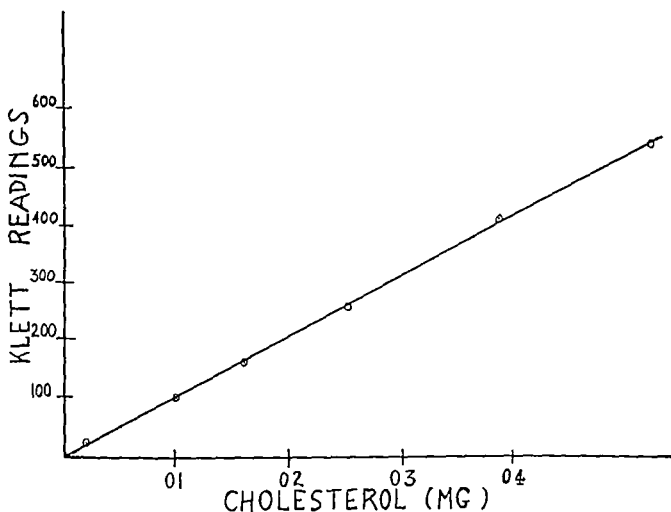


Fig 1—Standard curve plotting milligrams of cholesterol against Klett readings Total volume 3.1 milliliters

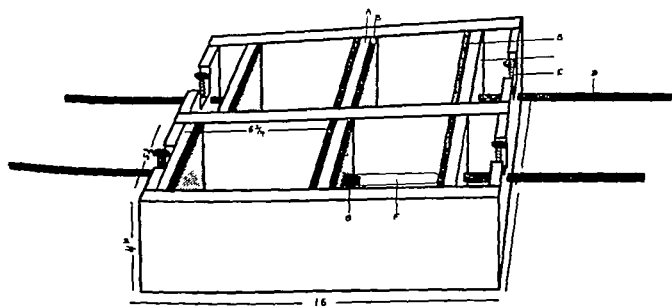


Fig. —Container with four compartments for shaking 150 to 200 stoppered test tubes  
 A wooden partition B rubber facing C movable wooden partition D metal rod E clamping screw  
 F test tube G rubber stopper held in place

By moving the plungers forward and fixing them in place with set screws, the rubber-stoppered test tubes are held tightly in place as they are shaken back and forth

#### REAGENTS

10 per cent sulfuric acid (10 Gm of concentrated  $\text{H}_2\text{SO}_4$  made up to 100 ml)

Chloroform, analytical grade

Acetic anhydride, analytical grade

Cholesterol standard, 0.2 mg per millimeter Forty milligrams of cholesterol recrystallized from acetone and dried are dissolved in less than 200 ml of acetic anhydride in a volumetric flask at  $60^\circ\text{C}$ , when completely dissolved, the solution is made up accurately to the 200 ml mark

Recovery solution, 0.025 mg per millimeter Fifty milligrams of cholesterol are dissolved in 200 ml of chloroform, 10 ml of this solution are diluted to 100 ml

Fat free stoppers No. 0 are allowed to soak in chloroform for about half an hour and then dried with gauze This is repeated each time before use

#### PROCEDURE

To a 15 ml test tube is added 0.1 ml of blood serum, to this is added 4 ml of 10 per cent sulfuric acid The acid should be blown from the pipette with force in order to prevent the protein from adhering to the walls of the tube To this are added exactly 4 ml of chloroform from a burette For the recovery study, 4 ml of the recovery solution are added instead of the 4 ml of chloroform The tube is then stoppered tightly and shaken in a shaking machine for twenty to thirty minutes It is then centrifuged The supernatant acid and the protein at the interface are then aspirated off with the aid of water suction. A 2 ml aliquot is now taken from the remaining chloroform, to which is added 1 ml of acetic anhydride This is done by touching the tip of the pipette lightly to silicone grease, inserting the pipette into the chloroform, blowing off the silicone grease, and aspirating the chloroform with a water aspirator The solution is mixed and 2 drops (0.04 ml) of concentrated sulfuric acid are added and the mixture is shaken vigorously The color is allowed to develop for seven minutes in a water bath at  $25$  to  $26^\circ\text{C}$ , in the dark, and then read on a Klett Summerson photoelectric colorimeter with a No. 60 filter, or on the Coleman spectrophotometer using 625  $\text{m}\mu$  as the absorption beam

#### STANDARD AND BLANK

The standard is developed and read at the same time as the unknown It consists of 1 ml of the 0.2 mg per millimeter cholesterol standard, plus 2 ml of chloroform and 0.04 ml of concentrated sulfuric acid It is developed and read in the same manner as the unknown

The blank consists of 1 ml of acetic anhydride, 2 ml of chloroform, and 0.04 ml of concentrated sulfuric acid

#### CALCULATIONS

The concentration of the cholesterol in the unknown may be read off the standard curve Since this curve is a straight line the formula below will yield the cholesterol values in milligrams per cent when 0.1 ml of serum is used

$$\frac{\text{Density of unknown}}{\text{Density of standard}} \times 0.2 \times \frac{100}{0.05} = \text{mg \% of cholesterol}$$



## RESULTS

The results obtained are listed in Table I. It is apparent that the results compare favorably with those obtained by extraction with Bloor's reagent. Table II indicates the amounts of cholesterol recovered when known amounts are added, as indicated under "Procedure."

TABLE I. COMPARISON BETWEEN THE KAYE METHOD AND THE AUTHORS' METHOD  
(The values are expressed as milligrams per 100 c.c. of serum. Each value is the average of duplicates.)

SAMPLE	NEW METHOD	KAYE METHOD	SAMPLE	NEW METHOD	KAYE METHOD
1	228	219	6	184	182
2	300	289	7	268	269
3	189	189	9	139	139
4	309	307	9	217	216
5	202	195	10	165	262

TABLE II

NUMBER OF DETERMINATIONS	CHOLESTEROL ADDED	MEAN AMOUNT	AVERAGE DEVIATION FROM MEAN
18	0.1 mg	0.098	$\pm 0.002$

See Procedure for details.

## SUMMARY

A convenient method is described for determining total cholesterol in sera employing a chloroform acid extraction.

The results are available within forty five minutes after the beginning of the determination.

The results are comparable to those obtained with Bloor's method of extraction.

This procedure is recommended where large numbers of determinations are required in a short time.

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# METHODS FOR THE CHEMICAL DETERMINATION OF CORTICOSTEROIDS IN URINE AND PLASMA

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THE methods here described derive from one suggested in abstract<sup>1</sup> but found inadequate. The underlying principles are extraction and partial separation of neutral steroids from urine or plasma and oxidation of the  $\alpha$  ketol or  $\alpha$  glycol side chains of the  $C_{21}$  steroids of adrenal cortical origin with periodic acid<sup>2</sup> followed by colorimetric measurement of the formaldehyde formed in the oxidation<sup>3</sup>. Oxidation and colorimetry proceed in the manner described for determination of mannitol<sup>4</sup> with the exception that formaldehyde is distilled from the reagent mixture before treatment with the chromogenic reagent. This modification has been described independently<sup>5</sup> for determination of urinary cortisol in a procedure which corresponds generally with the one here described.

The greater specificity of a procedure which depends on periodic oxidation as compared with sugarlike reduction of copper reagents is now accepted by many workers in the field. It is therefore desirable to present together analogous methods for estimation of corticosteroid in both urine and plasma which include this principle.

## APPARATUS

All glass, standard taper flasks, distillation apparatus and semimicro flasks and condenser  
Tall (200 x 20 mm) glass test tubes calibrated at 10 or 12.5 cc, Lewis and Benedict blood sugar tubes are suitable  
Spectrophotometer\* or other suitable photocolometric apparatus  
Automatic pipette (5 cc) for rapid, free delivery of chromotropic acid reagent

## REAGENTS

Solvents (1) Chloroform, chemically pure, redistilled (2) Glacial acetic acid, Baker special (emphysema free) (3) Alcohol ether 3 volumes 95 per cent alcohol to 1 volume of ethyl ether (4) Petroleum ether, boiling point, 30 to 60° C (5) Acetone, chemically pure

Sodium sulfate, anhydrous

Sodium hydroxide, 0.1N

Saturated aqueous solution of magnesium chloride

Periodic acid reagent Potassium periodate, 0.03M in 0.25M sulfuric acid

Stannous chloride reagent Prepared daily by dissolving about 1.4 Gm stannous chloride in 50 cc of 25N HCl. The solution is titrated to a starch end point with periodic acid reagent and adjusted so that 10.2 cc of periodic acid reagent oxidizes 10 cc of stannous chloride reagent

Chromotropic acid reagent Dissolve 0.2 Gm of chromotropic acid (1,8 dihydroxynaphthalene sulfonic acid) in 4 cc of water in a 100 ml volumetric flask and make up to volume with 15M sulfuric acid. The solution is prepared daily

Approximately 9M sulfuric acid

From the Research Division of the Cleveland Clinic Foundation

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\*Coleman Junior Clinical Spectrophotometer (Model 6A)

## PROCEDURE

*Urinary Corticosteroid—*

*Collection and Extraction* A twenty four or twelve hour night urine sample is collected. Depending on the adequacy of refrigeration and the speed of beginning extraction no preservative need be added or there may be added 5 cc of chloroform or 1 cc of 1 per cent aqueous Merthiolate (Lilly).

A four or six hour aliquot of the sample is brought to about pH 10 with concentrated HCl, 100 cc of chloroform are added. The mixture is shaken once and refrigerated until analyzed.

The urine is extracted four times with 100 cc portions of chloroform by shaking for fifteen minutes with each portion. The urine layer is discarded from a separatory funnel and the combined extracts and emulsions are centrifuged. The remaining urine layer is discarded. The extracts and emulsions are combined and dried by addition of sodium sulfate. The clear supernatant extract is filtered through glass wool.

This extract is chilled, washed twice with 0.1 volume of cold 0.1N NaOH and once with water. Each washing is back extracted with an equal volume of chloroform. These extracts are added to the washed chloroform extract and the NaOH and water are discarded.

The washed chloroform extract is evaporated in vacuo at less than 50°C in an all glass distillation apparatus to a volume of about 10 cubic centimeters. At this point the extract is divided in two equal portions and each is quantitatively transferred by rinsing with small volumes of chloroform into round bottomed distillation flasks of 25 cc capacity. Evaporation is continued to dryness.

*Colorimetric Assay* (1) Oxidized sample. The dried residue from one of the two flasks is dissolved with 0.5 cc of glacial acetic acid. When the flask residue is thoroughly wetted, 8.5 cc of water are added and to the mixture 0.5 cc of periodic acid reagent is added. The mixture is allowed to stand at room temperature for thirty minutes when oxidation is arrested by addition of 0.5 cc of stannous chloride reagent. (2) Unoxidized sample. The residue is brought into solution as described and made up with water to 9 cubic centimeters. Then 0.5 cc of stannous chloride reagent is added followed immediately by 0.5 cc of periodic acid reagent. (3) Blank on reagents. Oxidized and unoxidized reagent blanks are prepared from volumes of all reagents equal to those used in analysis.

The distillation flask is attached to a semimicro condenser. The elongated outlet tube of the condenser is placed under the meniscus of 10 cc of water in a 10 cc volumetric flask. About 8 cc of distillate are collected by careful heating over a microburner. The distillate is then made up to volume with water.

Three cubic centimeters of distillate are placed in a tall glass test tube and 5 cc of chromotropic acid reagent are rapidly mixed in. The tube is placed in a boiling water bath for thirty minutes. At the end of this time it is rapidly cooled to 25°C made up to volume (10 or 12.5 cc) with 9M sulfuric acid and stabilized to 25°C in a water bath. Color density (D) is measured at 570 microns.

*Calculation* The color density due to liberation of formaldehyde from the unoxidized sample is subtracted from the color density of the oxidized sample. From this is subtracted the corresponding color density obtained from the difference between oxidized and unoxidized blanks. The resultant color density represents formaldehyde liberated from corticosteroid like substances in the extract of urine. The amount present is found by reference to a calibration curve prepared from oxidation and colorimetry of desoxycorticosterone in 20 per cent alcohol. The result is expressed as milligrams corticosteroid per twenty four hours.

*Plasma Corticosteroid—*

*Collection and Extraction* The plasma from about 50 cc of fresh heparinized blood is added drop by drop with stirring to 5 volumes of 3:1 alcohol ether. The protein precipitate is collected on a sintered glass filter and thrice extracted by washing with 50 cc portions of alcohol ether. The filtrates are combined and the protein residue is discarded.

The combined alcohol ether extract is evaporated under reduced pressure at less than 50°C to a volume of about 30 cubic centimeters. To the substantially aqueous residue 95

per cent alcohol is added to make a solution containing about 70 per cent alcohol. This solution is extracted three times with equal volumes of petroleum ether. The petroleum ether washings are then discarded.

The clear, aqueous alcohol extract is evaporated in vacuo to a volume of about 5 cubic centimeters. Fifty cubic centimeters of acetone are added to the residue and 5 drops of saturated magnesium chloride reagent are stirred in. The mixture is allowed to stand in the cold for at least one hour. The clear, supernatant acetone extract is decanted through a sintered glass funnel. The gelatinous precipitate of phosphatide remaining is dissolved in portions of water and 95 per cent alcohol of 3 and 15 cc volume respectively. The solution of phosphatide is then evaporated in vacuo to a volume of about 1 cc, when the precipitation with acetone and magnesium chloride is repeated as described. The acetone extracts are combined and brought to dryness in vacuo. Should the residue from this evaporation have any considerable bulk it should be dissolved in alcohol and water and the precipitation repeated again.

The all but imperceptible residue from the acetone extract is taken up in 50 cc of chloroform and chilled. The cold extract is then washed with cold 0.1N NaOH in the manner described for urine extract. It is then dried by addition of sodium sulfate, filtered through sintered glass, and divided into two samples of equal volume. These samples, to be oxidized and not oxidized respectively, are brought to dryness in 25 cc distillation flasks as described.

Colorimetry and calculation are done in the manner described for urine. The result is expressed as milligrams per 100 cc of plasma.

## RESULTS

*Urinary Corticosteroid*—The formaldehyde-forming content of adrenal cortical extract added to samples of urine and carried through the procedure was recovered in a proportion which averaged 80 to 90 per cent. Values obtained from analyses of twelve-hour night urine in ten normal male and female subjects

TABLE I AMOUNT OF CORTICOSTEROID LIKE SUBSTANCE PRESENT IN URINE OF NORMAL MALE AND FEMALE SUBJECTS

MALE SUBJECT	MG /24 HR.	FEMALE SUBJECT	MG /24 HR.
1	0.77	1	1.43
2	1.41	2	.46
3	1.66	3	1.63
4	1.33	4	.76
5	1.29	5	1.09
6	1.00	6	1.36
7	0.94	7	.28
8	0.72	8	.43
9	1.57	9	.32
10	0.81	10	.65
Mean	1.15		0.84
	±0.32		±0.29
Mean for whole group	0.995 mg /24 hr		

These values were obtained from analysis of twelve-hour night urine and are expressed as milligram of desorycorticosterone per 24 hours.

are shown in Table I. Redeterminations on twenty-four hour urine specimens from eight of these subjects show no difference from the mean value found with night urine nor any consistent variations between day and night corticosteroid output. Values found in various abnormal situations and conditions are listed in Table II. Deviations from the normal in the latter series correspond well with clinical prediction. The values found are comparable to those obtained by Daughaday, Jaffe, and Williams,<sup>6</sup> and the methods, as noted, differ only in certain particulars.

TABLE II OBSERVATIONS ON URINARY CORTICOSTEROID LIKE SUBSTANCES IN VARIOUS CLINICAL CONDITIONS

PATIENT	SEX	DIAGNOSIS	TREATMENT	RESULT (MG /24 HR )
1	M	Malignant hypertension		0.91
			Pyrogen 3 days	2.34
			Pyrogen 6 days	4.4
			Pyrogen 9 days	3.5
2	F	Malignant hypertension		1.54
			Pyrogen 1 day	2.27
3	F	Malignant hypertension		1.74
			Pyrogen 1 day	5.1
4	F	Addison's disease	DCA cortical extract	0.4
5	F	Addison's disease	DCA cortical extract	0.4
6	F	Addison's disease	DCA cortical extract	0.4
7	M	Addison's disease	DCA cortical extract	0.1
8	F	Cushing's syndrome		2.7
9	F	Cushing's syndrome		3.3
			Exploration adrenal	10.8
10	F	Pseudo hermaphrodite		1.1
11	F	Arrhenoblastoma (?)		1.4
12	F	Acromegaly		0.91

*Plasma Corticosteroids*—Recovery of the formaldehyde forming substances in adrenal cortical extracts added to plasma ranged from 85 to 100 per cent in six determinations. Values found in the venous blood of normal human beings and in normal dogs are listed in Table III. The means in the two groups were 0.25 mg per 100 cc of plasma.

TABLE III CORTICOSTEROID CONTENT OF BLOOD PLASMA

MALE SUBJECT	MG PER 100 CC	FEMALE SUBJECT	MG PER 100 CC
<i>Normal Human Beings</i>			
1 a	0.42	1	0.34
b	0.38		
2 a	0.20	2	0.20
b	0.11		
3	0.23	3	0.13
4	0.16	4	0.42
5	0.14		
<i>Normal Dogs</i>			
0.74	0.38	4.47	0.25
4.45	0.12	5.12	0.12
4.73	0.27	0.12	0.50
4.84	0.21		
5.03	0.38		

Variations of the level were found in normal male human Subject 1 in a value of 0.98 mg per 100 cc thirty six hours after a second and third degree burn. This value fell to 0.38 mg per 100 cc on the tenth day. Dog 0.12 yielded levels of 0.30 before and 0.6 mg at twenty four hours after administration of a bacterial pyrogen. Concurrent urinary corticosteroid values in this animal were 0.15 mg per twenty four hours during the three day period before pyrogen injection and 0.38 mg per twenty four hours in the three days after treatment. Variations in plasma level thus correspond with clinical prediction.

The validity of the method is further confirmed in experiments conducted with the assistance of Dr. John Reinhard. In these the left adrenal efferent vessels of dogs were ligated and a segment of the lumboadrenal vein was isolated.

by ligatures where it passes under the gland. A plastic catheter was inserted into the vein and brought out through the abdominal incision which was closed. The operations were done under pentobarbital anesthesia and after administration of heparin. Adrenal venous blood was then collected over varying intervals. Blood loss was partially compensated for by transfusion of whole dog blood, of gelatin, and of washed red blood cells after separation of plasma.

The courses of three such experiments are shown in Fig 1, which demonstrates the levels of corticosteroid output in the venous plasma of the left adrenal

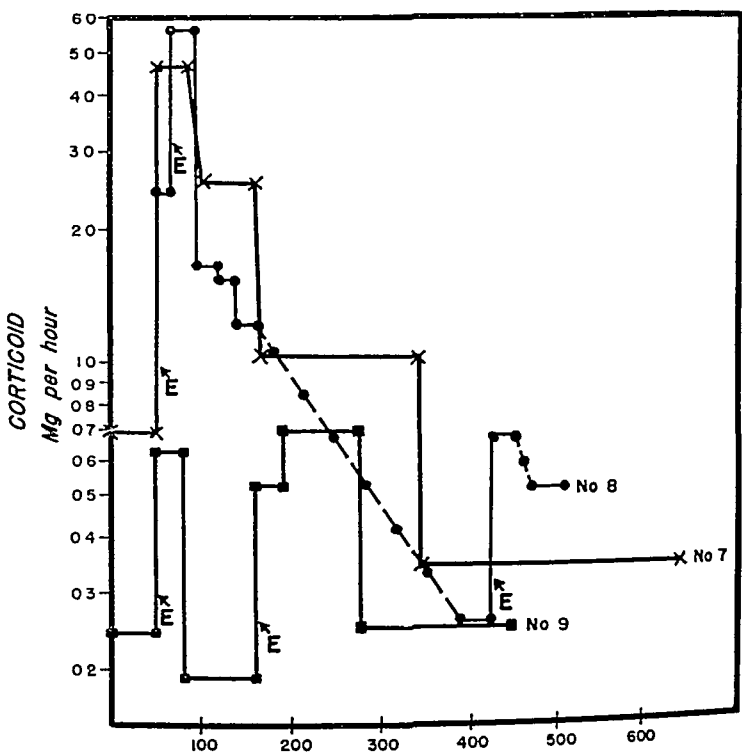


Fig 1—Output of corticosteroid (corticoid) into left adrenal venous plasma in three dogs at varying intervals after cannulating the isolated lumbo adrenal vein. Effect of injection of epinephrine (E) time of injection is indicated by arrows. Abscissa minutes after starting collection

TABLE IV CORTICOSTEROID OUTPUT FROM ADRENAL VEIN IN DOGS, EFFECT OF EPINEPHRINE, PERIPHERAL PLASMA LEVELS

EX PERI MENT	TIME OF COLLECTION (MIN)		ADRENAL VENOUS PLASMA				PERIPHERAL PLASMA (MG PER 100 CC)		EPI NEPH RINE
			CONCENTRATION (MG PER 100 CC)		OUTPUT (MG PER HR)				
	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	
3	0 75	76 150	4 8	3 5			0 4	0 9	0 7
3b	0 38	53 70	2 9	3 6	0 64	0 83	0 55	0 76	0 3
7	0 55	60 80	3 3	5 6	0 69	4 7	0 33	0 65	0 3
8	51 61	61 76	2 0	4 45	2 4	5 5	0 93	0 75	0 2
9	393 413	414 433	0 24	0 75	0 25	0 69		0 44	0 1
	0 60	61 80	1 0	1 5	0 24	0 63	0 43	0 7	0 2
	160 185	186 216	0 88	1 1	0 19	0 52	0 49		

Corticosteroid content of adrenal venous plasma at intervals indicated by time of collection in minutes before (A) and after (B) injection of epinephrine into the femoral vein. Output is calculated by multiplying plasma concentration by adrenal venous plasma flow

gland in milligrams per hour and the effects thereon of administration of epinephrine in doses of 0.2 to 0.5 mg into the femoral vein. Concentrations of corticosteroids in adrenal and peripheral venous plasma and the steroid outputs as well as simultaneous measurements in peripheral blood are listed in Table IV. The concentrations found in the adrenal venous plasma were higher than those obtaining in peripheral blood. The steroid output in these experiments averaged 0.85 mg per hour at the outset. In four of six experiments in which measurements were made the increased corticosteroid output caused by epinephrine was reflected in an increase in the concentration present in peripheral blood. However, we direct attention to these experiments (which will be reported in more detail) to confirm the method proposed rather than to deal with physiologic variations in the adrenal steroids.

#### DISCUSSION

*Urinary Corticosteroid*—Acidification of urine before extraction follows the recommendation of Heard, Sobel, and Venning.<sup>7</sup> Extraction is carried on four times to yield satisfactory recoveries of added adrenal cortical extract. Thus recoveries with two, three, and four extractions are 31, 65, and 94 per cent respectively.

The crude chloroform extract is washed with sodium hydroxide in the manner of Talbot, Salzman, Wixom, and Wolfe.<sup>8</sup> The washing reduces the bulk of the residue and thus reduces the probability of nonspecific contamination of the material which is to be oxidized. Other data<sup>8</sup> suggest that this step is not essential. However, on several occasions the residue from washing with water and alkali has been shown to contain small but significant amounts of chromogenic material.

The residue from the washed chloroform extract is dissolved in glacial acetic acid because of the good wetting quality of this reagent. The residue is not partitioned with benzene and water as was proposed<sup>1</sup> or as recently described because of the incompleteness of water extraction of certain steroids from benzene (Heard and Sobel<sup>9</sup>) and because the benzene-water partition of adrenal cortical extract yields a ratio of color density in benzene as compared with water of about 0.65.

Distillation of formaldehyde is done because of interfering colors which form during the heating of urinary or adrenal cortical extracts in the strong acid of the chromotropic acid reagent. The distillate is not received in sulfite as in the method of Daughaday, Jaffe, and Williams<sup>5</sup> because formaldehyde can be quantitatively recovered in water. It is important to distill a sufficient volume. With distillate volumes of 4.5, 5.0, 5.5, 8.0, and 8.5 cc, the respective recoveries of formaldehyde are 63, 75, 78, 98, and 101 per cent.

Mason<sup>6</sup> developed an almost identical procedure. In his method as in ours fractionation of the steroid extract with Girard T reagent<sup>8</sup> is omitted because results on unfractionated extracts parallel those obtained with the more tedious fractionation. It may be of interest to note that, in our hands, the ketonic

<sup>1</sup>Minn. We acknowledge the cooperation and advice of Dr. H. L. Mason, Mayo Clinic, Rochester.

fraction in urinary extracts accounted for about one-third of the total oxidizable steroid \*

*Plasma Corticosteroid*—The desirability of determinations of plasma corticosteroid is suggested by the fact that the amount measured in human urine averages about 0.05 per cent of the amount circulating through the kidneys. Rephrased, the urinary plasma clearance of corticosteroid is only 0.11 cc per minute. The apparent threshold of excretion is therefore very high, as indeed it should be for a material so important in bodily function. However, the inference is that under certain conditions measurements of plasma corticosteroid may have greater diagnostic and physiologic value than measurements in urine. This is especially true in experiments of brief duration, such as after injection of epinephrine.

The feasibility of such a determination was demonstrated by Hemphill and Reiss.<sup>10</sup> Their procedure measured corticosteroid by applying to plasma extracts the method of Talbot, Salzman, Wilcox and Wolfe,<sup>8</sup> while the method here presented has the advantages of the procedure described for urine extracts. As compared with the method of Hemphill and Reiss<sup>10</sup> the extraction process has been elaborated. Thus, we have found it essential to precipitate out phosphatide and, apparently because of entrapment of corticosteroid onto the phosphatide precipitate, the precipitation has to be repeated more than once. Further, an interfering chromogen is separated by washing plasma extract in 70 per cent alcohol with petroleum ether. Preliminary experiments indicate that it may be more convenient to make the first extraction by adding plasma to acetone containing 2 per cent chloroform. In a personal communication, Dr. O. M. Hechter<sup>7</sup> suggests that direct extraction of whole blood or plasma with chloroform is also satisfactory. Thus, while it is apparent that modifications can be made in the procedure, it is presented here in the form in which it has been most extensively tested.

As noted previously, observations on the corticosteroid output of the adrenal in dogs and the effects thereon of adrenalin are not the focus of this communication. Still it is of interest to note that the experiments listed here confirm the observations Vogt has made by bio-assay.<sup>11</sup> Thus, she estimated output of active steroid at about 230 cc Eucortone per twenty-four hours in a dog weighing 10 kilograms. Our estimate in terms of Upjohn adrenal cortical extract analyzed by oxidation and colorimetry would be about 88 cubic centimeters. Again, Vogt showed an increase of hormone output after injection of epinephrine. This estimate is confirmed in Fig. 1. In Table IV we also show that increased output due to epinephrine is often reflected in an increase in the corticosteroid content of peripheral as well as adrenal plasma.

As might be surmised, simple modifications enable the application of the plasma procedure to samples of tissue.

#### SUMMARY

Methods are presented for the estimation of corticosteroids in urine and plasma. The underlying principles are (1) extraction and partial separation

\*We would also note the helpful interest of Dr. Eleanor Venning of the Royal Victoria Hospital, Montreal, Canada.

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of neutral steroids from urine or plasma, (2) oxidation of the side chains of the C<sub>1</sub> steroids of adrenal cortical origin by means of periodic acid and (3) colorimetric measurement of formaldehyde formed in the oxidation. Values found in normal subjects are listed, together with illustrative clinical and experimental observations which indicate the application of these procedures.

The authors take pleasure in noting the skillful assistance of Mr Frank Ungar, B.A. M.S., in the development of the plasma procedure and of Mrs Lorraine Friedman, B.A. in the urine method.

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# QUICK MICROTECHNIQUES FOR THE IDENTIFICATION OF CULTURES

## I INDOLE PRODUCTION

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THE bacteriologist has always been handicapped in identification of organisms, as compared with other biologists, by the fact that the organisms with which he deals cannot be identified by their morphologic characteristics alone. The necessity of including cultural and biochemical characteristics has made identification a time-requiring procedure. Cultures need time to grow and to produce biochemical reactions.

In the diagnostic laboratory especially, the value of bacteriologic work definitely has been limited by the time required to isolate and identify the organisms from the patient. In addition, bacteriologic work often becomes expensive because of the time required and the materials and equipment needed. The chemist has partially solved the problem of expense by the development of semi-micro- and microtechniques.

This study of indole production was undertaken to see if microtechniques could be applied to the study of biochemical reactions of microorganisms and if, at the same time, results could be obtained in a shorter period of time.

The principle on which the test is based is the use of small amounts of media and large inoculums to allow the organisms to start growing without appreciable lag phases and to produce, in a short period of time, sufficient concentrations of biochemical products to be detectable if sensitive reagents are used. Studies have been made to determine the optimum conditions for indole production and detection by the microtechnique.

### DEVELOPMENT OF THE MICROTECHNIQUE

Tests proved to be most satisfactory with 1 ml quantities of medium in 10 by 75 mm tubes. Smaller tubes were not practicable because of difficulty in mixing the test solution with the medium.

For the detection of the indole, the Ehrlich, Kovács,<sup>1</sup> and Gore methods were tried. For the preliminary testing of these methods a medium containing 10 per cent tryptone and 0.3 per cent beef extract was used. Tubes of this medium were heavily inoculated with a stock strain of *Escherichia coli*. Tests for indole were performed on these tubes after periods of incubation at 37° C for from two and one-half to six hours. With the Kovács method a doubtful reaction was obtained in one of two tubes after three hours of incubation, and a strongly positive reaction was obtained in all the tubes that were incubated for four hours or longer. The other two methods were less sensitive, requiring

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at least one hour more of incubation to obtain comparable results. Because of these results and because its application is simplest Kovacs' method was selected for all subsequent work.

In the test, four drops of fresh Kovacs reagent are added to the 1 ml quantity of medium in the tube. The tube is shaken and the results are read after a few minutes. In the early part of the work the chloroform solubility test of Fellers and Clough<sup>3</sup> was used to check the specificity of the positive reactions. Since these reactions always proved to be specific this test was discontinued later. The Kovacs reagent must be fresh. Upon standing at room temperature its color changes from yellow to brown and it becomes less sensitive. It may be preserved for several days in the refrigerator. For the preparation of the reagent, various brands of amyl alcohol, isoamyl alcohol and isobutyl alcohol were tried. Of these the isoamyl alcohol proved to be superior, some of the amyl alcohols giving false colorations and the isobutyl alcohol failing to separate sharply from the medium.

After a study of a number of media two were selected for use in the micro technique. Medium 1 consisted of 1.0 per cent tryptone and 0.3 per cent beef extract, in distilled water. Medium 2 consisted of 0.03 per cent tryptophane, 0.1 per cent peptone, and 0.5 per cent  $\text{K}_2\text{HPO}_4$  in distilled water. It was found that in Medium 1 a salt mixture consisting of 0.1 per cent  $\text{NaCl}$ , 0.02 per cent  $\text{MgSO}_4$ , 0.01 per cent  $\text{CaCl}_2$ , and 0.1 per cent  $\text{K}_2\text{HPO}_4$  could be substituted for the beef extract without affecting the results. Since the salt mixture added to the complexity of the medium without adding to its productiveness it was not used in later tests of the technique. The addition of either beef extract or the salt mixture to Medium 2 did not affect the results obtained. For the peptone in Medium 2 Coleman and Bell Bacto peptone, Lacto proteose peptone, 3 Bacto tryptone and Bacto tryptose were tried. All gave equally good results.

The addition of 0.6 per cent agar to Medium 1 caused false colorations to be produced with the test reagent. A medium consisting of 1.0 per cent Bacto peptone and 0.3 per cent beef extract was found to be unsuitable for use in the micro technique.

Batches of Medium 1 and of Medium 2 with pH values varying from 7.0 to 8.0 were tried. Equally good results were obtained with pH values between 7.4 and 7.8. At pH 7.0 and pH 7.2 indole was formed as quickly as at the higher pH values but the amount formed was less. For example in one experiment indole was formed at all pH values between 7.0 and 7.8 in six minutes, either doubtful or weak reactions being obtained. After fifteen minutes of incubation all tubes with pH values between 7.4 and 7.8 yielded strongly positive reactions whereas those with pH values of 7.0 and 7.2 yielded doubtful or weak reactions. In the later use of the micro technique all media were adjusted to pH 7.4.

The testing of a large number of cultures with Medium 1 and Medium 2 indicated little difference in value for the two media. No discrepancies in results have been noted. With many cultures the time required for indole production in Medium 2 from tryptophane is about one third less than the time required

for production in Medium 1, from tryptone. This saving in time may not be considered sufficient to warrant the use of the more expensive Medium 2.

Quick production of indole depends upon rapidly obtaining the incubation temperature of 37° C. Preheating of the tubes of medium before inoculation shortens materially the time required for indole production. Water bath incubation instead of hot air incubation also helps. Usually if a culture will produce indole in two hours without preheating of the medium it will do so in from thirty minutes to one hour with preheating and in from eighteen to thirty minutes with water bath incubation. We have obtained indole production in six minutes with water bath incubation.

Absolute sterility has been found to be unessential with the microtechnique. In this study the tubes and the medium were always sterile but a relative absence of contaminants is probably all that is necessary. Cotton plugs do not need to be used. A small number of contaminants cannot multiply rapidly enough to affect the results of the test.

The size of inoculum is important. Inoculation of a tube with all the growth obtainable from a colony with a diameter of 2 mm will give good results. Inoculation with the larger amount of growth obtainable from an agar slant culture will yield somewhat quicker indole production. Inoculation with growth from a culture which is in the logarithmic period of development will give quicker results than inoculation from an older culture.

#### COMPARISON OF MICROTECHNIQUE WITH GNEZDA TECHNIQUE

For testing the accuracy of results obtainable with the microtechnique, comparative tests were made with the Gnezda<sup>4</sup> technique. For the Gnezda technique tryptone broth was used and incubation was at 37° C for four days.

Results with the two techniques were identical. Cultures which gave positive results were fifty-seven strains of coliforms, three strains of *Pseudomonas caviae*, two strains of *Proteus mirabilis*, and one strain each of *Proteus vulgaris* and *Shigella paradysenteriae*. Of these, two of the strains of coliforms yielded weakly positive results with the Gnezda technique after four days. All the other strains gave strongly positive results with both methods. Cultures which gave negative results were three strains of coliforms, nine strains of unidentified soil organisms, and one strain each of *Salmonella schottmulleri*, *Salmonella paratyphi*, *Salmonella enteritidis*, *Vibrio comma*, *Pseudomonas aeruginosa*, and *Pseudomonas fluorescens*. The majority of the strains of coliforms were fresh isolates and the tests were made with inoculums from the primary colonies on the selective media used for isolation. All other cultures used were stock strains.

The rapidity with which indole was formed in the microtechnique raised the question as to whether the indole was being formed because of growth of the organisms and enzyme production during incubation or because of preformed enzymes that were present in the large inoculum. In this connection we were interested in determining the effect of the medium from which inoculum was taken on the rate of indole formation.

From thirteen strains of coliform organisms, streaks were made on nutrient agar plates and on tryptone agar plates. After a twenty-four hour period of

incubation, tests for indole forming ability were made by the microtechnique with inoculums from both types of plates. Of the thirteen strains, ten gave positive indole tests after one and one half hours when the inoculums were taken from the nutrient agar plates. Of these, one gave a positive test after eighteen minutes, five after thirty six minutes, two after one hour and two after one and one half hours when the inoculums were taken from the tryptone agar plates. Thus growth of the inoculum culture on tryptone agar appeared, in the majority of cases, to increase the speed with which indole was produced.

Using the two strains of coliforms that had produced only traces of indole after two hours, an attempt was made to increase the rate or amount of indole formation by transferring for two generations on tryptone agar plates. No increase in rate or amount of indole formation occurred.

The laboratory strain of *Escherichia coli* was grown for four generations on a synthetic tryptophane free medium. At the end of this time indole determinations were made using colonies from the synthetic medium, from nutrient agar plates, and from tryptone agar plates as sources of inoculum. Again indole was formed more rapidly in the tubes that had been inoculated from colonies on the tryptone agar plates. Surprisingly however indole was formed slightly more rapidly in the tubes that had been inoculated from the synthetic medium than in those that had been inoculated from the nutrient agar plates.

From this series of experiments it may be concluded that the medium from which the inoculum is taken has only a minor effect on the results of the micro technique for the determination of indole producing abilities of bacterial cultures.

#### SUMMARY

A quick microtechnique for the determination of the abilities of micro organisms to produce indole has been described. Heavy inoculations are made into 1 ml quantities of medium in 10 by 75 mm tubes that have been preheated to 37° C. Either tryptone broth or a synthetic medium containing tryptophane has been found to be satisfactory. The tubes are then incubated at 37° C preferably in a water bath. Indole may be detected in the tubes upon the addition of 4 drop quantities of a fresh Kovacs solution which has been made with isoamyl alcohol as the solvent. The period of incubation that is necessary has been found to vary from six minutes to two hours depending upon the strain of organism, the age and size of inoculum and the method of incubation.

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# QUICK MICROTECHNIQUES FOR THE IDENTIFICATION OF CULTURES

## II FERMENTATIONS

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SUCCESS in developing a quick microtechnique for the detection of indole producing abilities of microorganisms (Arnold and Weaver<sup>1</sup>) led to an attempt to develop a similar technique for the demonstration of fermenting abilities of microorganisms. The principle on which the test to be reported is based is the same as that used in the test for indole production, namely the use of small amounts of media and large inoculums to allow the organisms to start growing without appreciable lag phases and to produce, in a short period of time, sufficient concentrations of biochemical products to be detectable if sensitive reagents are used.

### DEVELOPMENT OF THE MICROTECHNIQUE

The microtechnique was developed using cultures of the Enterobacteriaceae as test organisms. Thus far its use has been limited to this group of organisms.

Tests proved to be most satisfactory with 0.15 ml quantities of medium in 5 by 50 mm tubes. Larger tubes, 10 by 75 mm, containing 0.5 to 1.0 ml quantities of medium could be more easily handled, but their use in place of the smaller tubes approximately doubled the time required for the demonstration of fermentation.

Of a number of basal media that were investigated, all but one was discarded as unsatisfactory. Thioglycollate broth, nutrose solution, neopeptone solution, and neopeptone and tryptone solution were discarded because the rate of fermentation in them was too slow. A yeast extract medium was discarded because the yeast extract contained some substance or substances that would allow the production of both acid and visible gas by *Escherichia coli* cultures under the conditions of the microtechnique.

The following basal medium was selected for use: Difco beef heart infusion 7.5 per cent, proteose peptone 3, 1.0 per cent,  $\text{KH}_2\text{PO}_4$ , 0.1 per cent,  $\text{NaCl}$ , 0.5 per cent, distilled water, pH 7.0. Indicator: 5 ml of a 1.6 per cent alcoholic solution of bromocresol purple and 5 ml of a 1.6 per cent alcoholic solution of cresol red per liter of medium.

This medium proved to be free of fermentable substances when it was tested with strains of *E. coli*. Later it was found that controls of this basal medium that had been inoculated with *Aerobacter aerogenes* showed slight visible gas formation, although by the usual macrotechniques *A. aerogenes* could not be shown to produce any fermentation in the medium. It was therefore necessary

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to remove the fermentable substances from the beef heart infusion by fermentation with *A. aerogenes*. The beef heart infusion was dissolved in the distilled water. This solution was inoculated with 5 ml of a twenty four hour nutrient broth culture of *A. aerogenes*. It was then incubated at 37° C until bubbles ceased to appear approximately forty hours. The organisms were removed by passage through a filter pad\*. The remaining ingredients were then added to this infusion, and the resultant medium was adjusted to pH 7.0. This basal medium was then sterilized by autoclaving.

When the tests were to be run a sufficient quantity of a 20 per cent solution of the carbohydrate to be tested to produce a final concentration of 5 per cent was added to the basal medium. It was found that the carbohydrate solution had to be sterilized by filtration. The use of autoclaved solutions resulted in false reactions by the microtechnique even when the use of the same solutions in medium for tests by the macrotechnique did not. The final medium was then dispensed in the small tubes with capillary pipettes. In all the work reported upon in this study sterile pipettes and sterile tubes were used but it has been found that only relative sterility is necessary. Cotton plugs may be omitted. It was found that the time required for the test could be reduced by preheating the tubes of medium in the water bath previous to inoculation.

If inoculation is to be with liquid medium cultures or with suspensions of the organisms in saline the medium should be prepared in double strength to allow for dilution of the medium with the liquid of the inoculum.

The inoculum should be large. The time required for the test will be shortened if the culture from which the inoculum is taken is in the logarithmic growth period. Inoculation from growth on a solid medium with a platinum needle is satisfactory. In this study most of the inoculations were made with suspensions that had been prepared by emulsifying growth from an agar slant culture or from a colony in a small amount of sterile saline. Inoculations were made in this case by adding 0.15 ml quantities of the suspensions to the medium in the tubes by means of capillary pipettes.

The inoculated medium was then capped with a 3 mm layer of 1 per cent agar in distilled water to which indicator had been added in the proportion of 1 ml each of a 1.6 per cent alcoholic solution of biomeresol purple and a 1.6 per cent alcoholic solution of cresol red per liter. The indicator was added to the agar solution to detect any change in pH due to absorption of acid substances from the air during storage. When this precaution was not taken, the reading of the results was occasionally confused by a small area of acid reaction at the junction of the agar solution and the culture caused by an agar solution that had become acid. Less indicator was added to the agar than to the medium to make the junction of these solutions more discernible.

The capping is done with a capillary pipette. The melted agar is permitted to flow down the inside wall of the tube. Care must be taken to avoid bubbles. If bubbles form they can usually be eliminated from the agar medium interface by a gentle tapping of the tube.

The quickest results are obtained by incubation in a 37° C water bath. This is due to the fact that the medium reaches the incubation temperature much more quickly in the water bath than it does in a hot air incubator.

Acid production may first be noted by the production of a yellow color just beneath the agar cap. Readings must always be made by comparison with inoculated controls of the basal medium that do not contain the test carbohydrate. If this is not done, false results may be obtained because of transference of acid to the medium with the large inoculum. Gas production is evinced by the collection of bubbles at the agar-medium interface. Acid production usually may be detected before gas production, but the opposite is occasionally the case.

#### RESULTS WITH MICROTECHNIQUE

Results with the microtechnique were compared with those with the macrotechnique on five strains of *A. aerogenes*, five strains of *E. coli*, ten strains of paracoli, ten strains of *Salmonella*, three strains of *Eberthella typhosa*, five strains of *Shigella*, and seven strains of *Proteus* using glucose, mannitol, sucrose, lactose, and maltose. The macrotechnique was run using Durham fermentation tubes and nutrient broth base plus filtered sugar solutions. Final results were read after seventy-two hours. Two determinations were made with the microtechnique. In the first, the tubes were inoculated with suspensions obtained by emulsifying single colonies from nutrient agar plates in 1 ml quantities of sterile distilled water. In the second, the tubes were inoculated with heavy suspensions obtained by emulsifying growth from twenty-four hour cultures on brain-heart agar slants.

The essential features of the results are summarized in the following statements.

Identical results were obtained with the macrotechnique and with both sets of microtechnique determinations.

The use of a heavy inoculum materially shortens the time required to obtain results with the microtechnique. With the light inoculum, acid production was obtained in from 15 to 725 minutes and gas production in from 75 to 660 minutes. With the heavy inoculum, acid production was obtained in from 10 to 230 minutes and gas production in from 35 to 240 minutes.

The simplest fermentable substances were fermented more rapidly than were the disaccharides. For example, with the six strains of organisms that fermented all the substances that were tested and with the light inoculum, an average of 95 minutes was required for production of acid from glucose, 85 minutes from mannitol, 130 minutes from sucrose, 140 minutes from lactose, and 145 minutes from maltose.

#### COMMENT

The microtechnique yields results that are as reliable as those obtained with the usual macrotechniques. It not only yields quicker results but its use also results in a considerable saving in time and materials. The stock basal medium may be sterilized and stored in the refrigerator. Likewise, 20 per cent solutions



of the various fermentable substances may be sterilized by filtration and stored in the refrigerator, with proper precautions being taken against evaporation. When it is desired to test cultures, the fermentable substances and basal medium may be mixed in the proper proportions and the tests may be performed immediately. Only precautions to prevent excessive contamination in the process are necessary.

The microtechnique is usable under a variety of conditions. The length of the incubation period depends upon these conditions. The most important condition is the type and size of inoculum. The quickest results are obtained with heavy inoculums with logarithmic growth phase cultures that have been grown on a rich medium. On the other hand, reliable results can be obtained with single colonies from primary isolation plates. While the incubation period required in the microtechnique in the latter case is longer, the results are obtained much more quickly than if it were necessary to grow secondary cultures before the microtechnique tests were performed.

#### SUMMARY

A quick microtechnique for the demonstration of fermenting abilities of microorganisms has been described. It has proved to give reliable results with members of the Enterobacteriaceae. No tests have been run on other cultures.

Heavy inoculations are made into 0.15 ml quantities of medium in 5 by 50 mm tubes which have been preheated to 37° C. A beef heart infusion medium containing indicator is used as the basal medium. To it is added a sufficient quantity of sterile (filtered) 20 per cent solution of the fermentable substance to be tested to produce a 5 per cent solution in the final medium. The inoculated medium is capped with a 3 mm layer of a melted 1 per cent agar solution. The tubes are incubated at 37° C. Gas production is evinced by the collection of bubbles below the agar cap. Incubation periods of from 10 minutes to 12 hours, depending upon the size of inoculum and other factors, have been found to be necessary.

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# A TEST OF THE COAGULATION TIME OF BLOOD HEPARINIZED IN VITRO, STUDIES OF NORMAL SUBJECTS AND OF PATIENTS WITH INTRAVASCULAR THROMBOSIS

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IT IS known that the addition of a specific amount of heparin to a specific amount of human blood may produce a variable increase of the coagulation time among different individuals. This principle has been applied by others in an attempt to develop a test which might be of value as an indication of increased coagulability of the blood in patients who have or may have thrombosis.

In 1943 de Takats and Gilbert<sup>1</sup> described a heparin tolerance test. They injected 10 mg of heparin intravenously and determined the coagulation time of capillary blood drawn into capillary tubes before and at ten minute intervals after injection until the coagulation time returned to preinjection levels. With this test de Takats<sup>2</sup> noted that little or no increase of coagulation time occurred in patients who had undergone major surgical procedures or who had had coronary thrombosis, venous thrombosis, arterial embolism, or thromboangitis obliterans, while a definite increase occurred in normal persons. Although they do not detract from the importance of the principle involved, there are certain theoretic objections to the technique which was used, namely the method of testing the coagulation time that de Takats and Gilbert used is not generally considered to be as accurate and reproducible as other methods, the differences between the maximal coagulation times in normal persons and those with thrombosis were of the order of only a few minutes and possibly within the range of error of the method (Meyer),<sup>3</sup> and tests were done at room temperature.

Waugh and Ruddick<sup>4</sup> in 1944 described a test of coagulation time of blood heparinized in vitro. Their method consisted of adding 1 cc of venous blood to each of a series of test tubes in which there were increasing amounts of heparin from 1 unit (0.009 mg) to 7 units (0.063 mg). The chief practical objections to this method are that it is time consuming and that too many tubes requiring accurate mixtures of blood and heparin are necessary for each test. Waugh and Ruddick did their tests at room temperature.

Hagedorn<sup>5</sup> repeated de Takats' heparin tolerance test but administered a larger amount of heparin, 25 mg, and determined coagulation times on venous blood (Lee-White method). He found that only two coagulation times were important, namely the one before injection and the one ten minutes after injection. Significant differences were found between the responses of normal

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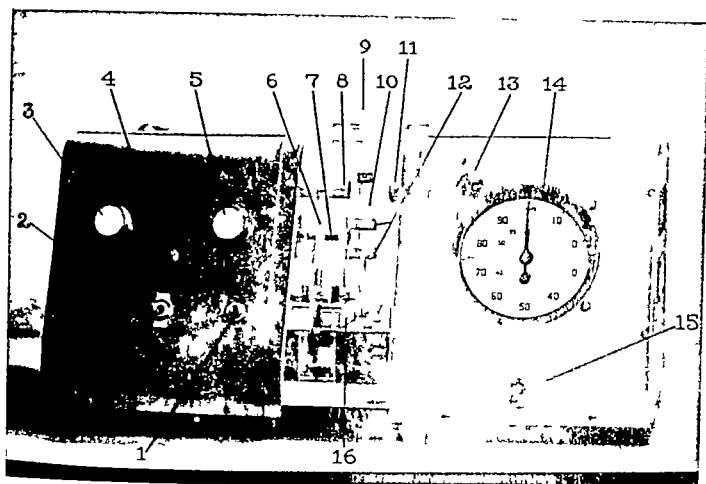


Fig 1—Front view of coagulochronometer 1 access to adjusting screw for varying sensitivity of photoelectric cell 2 motor switch 3 green light 4 push button to permit continuous rotation of test tube 5 red light 6 thermometer 7 motor shaft 8 tube transmitting beam of light to photoelectric cell 9 handle of plastic door permitting access to chamber for insertion and removal of test tube 10 test tube to hold blood being tested 11 tube to transmit beam of light from source of light 12 clamp for test tube 13 lever to reset stop clock 14 electric stop clock 15 switch for stop clock 16 heating unit and thermostat.

persons and the responses of a series of patients with intravascular thrombosis of various types. The differences seemed much greater than the possible error of the method of determining the coagulation time. Hagedorn also found a close correlation between the coagulation time of venous blood ten minutes after the intravenous injection of 25 mg of heparin and the coagulation time of 1 cc of venous blood added to a solution of 0.005 mg of heparin in vitro in each of a large series of both normal persons and patients with thrombosis. The chief objections to the Hagedorn method were that each test was time consuming and that tests were done at room temperature.

We wish to report the results of a coagulation test of venous blood heparinized in vitro in which we have attempted to increase the accuracy of the method and at the same time to simplify the technique so that only a minimal amount of time is expended in the performance of each test. In this test we have utilized the following equipment: 1 Barker coagulochronometer, 2 syringe adapted for accurately measuring and immediately mixing blood with a heparin saline solution, a box to maintain a constant air temperature of 37° C, Pyrex test tubes, venipuncture needles, and a solution of heparin.

The Barker coagulochronometer<sup>7</sup> (Fig 1) is a machine that automatically records the coagulation time of whole blood or heparinized whole blood. The machine consists essentially of a photoelectric cell which operates a sensitive

relay, a light source for the cell, an electric motor which rotates at the rate of 1 revolution per minute, a horizontal shaft fitted with a test tube clamp, and an electric stop clock. Blood is placed in a test tube which is closed with a rubber finger cot or cork and the test tube is placed in the clamp where it is grasped by its midportion. As the test tube rotates, its top passes through the beam from the light source. As long as the blood is fluid, it runs to the lower end of the tube with each half-revolution. When the blood coagulates it adheres to the lower end of the tube, and when that end of the tube rotates upward to the vertical position, the blood clot interrupts the light beam and breaks the electrical circuit operating the motor and stop clock. Thus the coagulation time is automatically recorded. This machine is primarily a labor-saving device, since it eliminates frequent tipping of the tube by hand to determine the end point. Also it tips the tube at a constant rate and to a constant degree.

To insure direct and accurate mixing of the heparin and blood, a syringe was devised by one of us.<sup>8</sup> This syringe, when fitted with a 21 gauge,  $1\frac{1}{2}$  inch (3.8 cm) venipuncture needle, holds exactly 1 cc of heparin in a saline solution. Venipuncture was then performed with this syringe. By means of a spring release mechanism, exactly 1 cc of blood could be withdrawn into the heparin-saline solution. The blood and the heparin solution were mixed immediately in the syringe.

Whittaker<sup>9</sup> demonstrated that heparinized blood must be kept at a constant temperature to obtain accurate coagulation times. In our early studies the coagulochronometer was kept in a cabinet in which the air temperature was maintained at 38° C. Later the midportion of each coagulochronometer was enclosed in transparent plastic and the air surrounding the test tube containing the blood was kept at 37° C by means of a heating element, thermostat and fan inside the closed space.

The solution of heparin was prepared by diluting Abbott's solution of heparin (10 mg per cubic centimeter) with 0.9 per cent solution of sodium chloride so that 1 cc contained 0.006 mg of heparin. This solution was prepared under sterile conditions and stored in vaccine bottles sealed with rubber diaphragms. All glassware was thoroughly cleaned and dried in drying ovens. When not in use, equipment was covered to avoid contamination with dust particles.

In performing a coagulation test with heparinized blood, a 21 gauge,  $1\frac{1}{2}$  inch (3.8 cm), steel venipuncture needle was attached to the syringe and 1 cc of heparin-saline solution was drawn into the barrel of the syringe. A tourniquet was applied to the subject's arm and as soon as one of the cubital veins became distended it was punctured. A finger release spring on the syringe was then depressed and the plunger was pulled back until it stopped. In this way exactly 1 cc of whole blood was added directly to the 1 cc (0.006 mg of heparin) of heparin-saline solution. Care was taken that the venipuncture was neat and that no air entered the heparin-saline-blood mixture. The tourniquet was released and the needle was withdrawn from the vein. The needle was removed from the syringe and the contents of the syringe were gently poured

down the side of a Pyrex glass tube 8 mm in diameter and 100 mm long. The test tube was sealed with a rubber finger cot and gently inverted to mix its contents. The test tube was then placed in the test tube holder of the coagulochronometer and the time clock and the motor were started. When the blood coagulated, it broke the light beam thus stopping the motor and the clock. The coagulation time was read from the clock dial. Hereafter in this paper the time is referred to as the heparin coagulation time.

After the blood had been withdrawn from the vein there was a delay of about one minute before it could be placed in the machine and the time clock started. Since this delay was short and a relatively constant factor in all of our tests we do not feel that it constitutes a significant source of error in interpretation of results.

It was our experience in determining coagulation times with this technique that if coagulation did not occur within thirty minutes a satisfactory end point rarely occurred. It appeared that the constant rotation of the tube ultimately produced defibrination of the blood. Thus the results of all the tests could be roughly divided into two groups: those in which coagulation occurred in less than thirty minutes and those in which coagulation did not occur in thirty minutes.

To determine the variability of the heparin coagulation test repeated tests were done on each of sixteen subjects in whom the first heparin coagulation time was less than thirty minutes. In each subject three successive separate blood samples were drawn according to the described technique and each sample was placed in a different coagulochronometer. The results are shown in Table I. The variability measured as the standard deviation is shown

TABLE I RESULTS OF THREE SUCCESSIVE HEPARIN COAGULATION TESTS DONE ON THE SAME INDIVIDUAL WITH THREE SUCCESSIVE VENIPUNCTURES

SUBJECT	HEPARIN COAGULATION TIME (MIN)				
	TEST			MEAN	STANDARD DEVIATION
	1	2	3		
A	1	14	18	15.0	.6
B	14	11	17	14.0	3.0
C	14	12	17	14.3	.5
D	20	24	18	20.7	3.1
E	25	28	22	25.0	3.0
F	18	12	19	16.3	3.8
G	15	16	15	15.3	0.6
H	10	14	15	13.0	2.6
I	18	24	20	20.7	3.1
J	17	20	15	20.7	4.0
K	25	17	26	22.7	4.9
L	20	21	19	20.3	1.5
M	16	18	15	16.3	1.5
N	12	11	20	14.3	4.9
O	25	27	No Cl	26.0	1.4
P	17	15	18	16.7	1.5
Total series				18.0	2.7

minutes Subjects in whom the first determination of coagulation time was less than thirty

† No coagulation.

for each case. For the sixteen subjects the average standard deviation was 27 minutes, giving a significant variability of plus-minus 54 minutes\*.

Five successive blood samples were drawn from five individuals in whom the first heparin coagulation time was more than thirty minutes. In none of the subsequent tests on these subjects was the heparin coagulation time less than thirty minutes.

The results of our studies of heparin coagulation time in control groups and in diseased states are shown in Table II. The second group in Table II consisted of patients who had been in bed from six to seventy days because of various diseases, but who did not have clinical evidence of intravascular thrombosis. In the cases of thromboangitis obliterans the disease was severe enough to require hospitalization. In each of the cases of arteriosclerosis obliterans the patient had been hospitalized because of ischemic ulcers or gangrene, but in no case had there been a recent arterial occlusion.

TABLE II DISTRIBUTION OF HEPARIN COAGULATION TIME IN VARIOUS CONDITIONS

DIAGNOSIS	TOTAL INDI- VIDUALS TESTED	HEPARIN COAGULATION TIME (MIN.)						
		30 or longer		20-29		10-19		LESS THAN 10
		NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER PER CENT
Normal ambulatory subjects	50	41	82	2	4	7	14	0
Bed patients without thrombosis	50	39	78	4	8	7	14	0
Thromboangitis obliterans	14	8	57	2	14	4	29	0
Arteriosclerosis obliterans	20	8	40	3	15	9	45	0
Recent venous thrombosis or pulmonary embolism	19	2	11	5	26	2	11	10
Acute peripheral arterial occlusion	3	1	33	0	0	0	0	2

In each case of thrombophlebitis or pulmonary embolism in Table II, the diagnosis was definitely established clinically and blood samples were drawn within one to seven days after the clinical onset of thrombosis. Fifty-two per cent of these patients had coagulation times less than ten minutes, this rapid coagulation time was not observed in any of the control subjects whom we studied.

We also had an opportunity to study three patients with acute arterial occlusion in the extremities shortly after the occlusion occurred. In one patient the heparin coagulation time was thirty minutes or more, but in two the time was less than ten minutes.

#### SUMMARY

We have described a technique for the determination of the coagulation time of 1 cc of venous blood mixed with 1 cc of 0.9 per cent solution of sodium chloride containing 0.006 mg of heparin. The technique utilized a

\*Plus-minus 2 times the standard deviation is a generally accepted range for statistically significant variation.

coagulochronometer (a machine for automatically recording the end point on a stop clock) and a syringe which was specially devised to permit first the withdrawal of exactly 1 cc of the heparin saline solution from a bottle and then of exactly 1 cc of blood from a vein into the heparin saline solution. All tests were done in an environmental temperature of 37° C.

Coagulation times by this method were less than thirty minutes in only 18 per cent of fifty normal ambulatory subjects and in 22 per cent of fifty bed patients without evidence of thrombosis. Coagulation times were less than thirty minutes in 53 per cent of thirty-four patients with chronic occlusive arterial disease of the extremities (thromboangitis obliterans or arteriosclerosis obliterans). Coagulation times were less than thirty minutes in 89 per cent and less than ten minutes in 52 per cent of nineteen patients with recent clinical venous thrombosis or pulmonary embolism. No coagulation times were less than ten minutes in any of the control subjects.

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# A CAGE WHICH LIMITS THE ACTIVITY OF RATS

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WITH THE TECHNICAL ASSISTANCE OF EMERY VAN HOOK

THE cage shown in Fig 1 was developed to restrain a rat during periods of days while an indwelling tube was in place. It has proved very useful in collecting urine through an indwelling cystostomy tube, for continuous or intermittent injection through a small plastic tube inserted into a vein, for collection of lymph from the thoracic duct, intestine, or liver over periods of days through a small indwelling plastic tube in a lymphatic vessel, and for other procedures

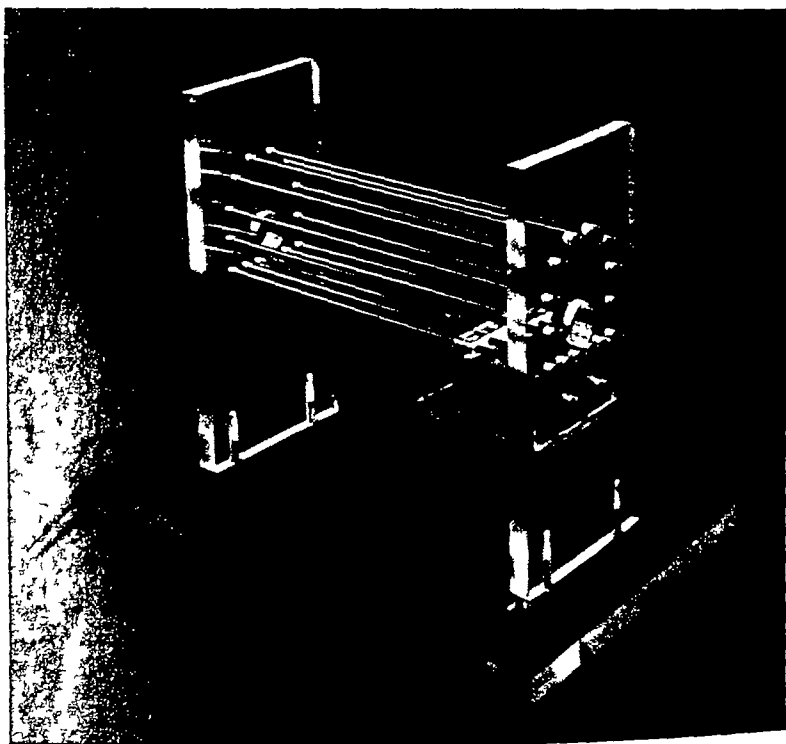


Fig 1—The assembled cage.

The cage is made of Lucite  $\frac{3}{8}$  inch thick, which may be worked with ordinary tools. The endpieces are 3 by 6 inches and the hole in each piece, which is  $\frac{5}{8}$  inch in diameter, accommodates a drinking fountain tube or the tail of the rat. Fourteen steel rods,  $6\frac{1}{2}$  inches long and  $\frac{1}{8}$  inch in diameter, set approximately  $\frac{1}{2}$  inch apart, constitute the floor and sides of the cage. These fit into slots drilled in one endpiece. Holes in corresponding positions in the other end piece are drilled through and threaded to accommodate small brass screws. The enclosed space,  $1\frac{3}{4}$  inches wide and  $1\frac{3}{4}$  inches high, accommodates a rat which weighs 200 grams. Additional holes and slots may be made in the endpieces so that the rods may be placed to fit larger or smaller rats. The food cup, which is placed under an opening in the floor, has been satisfactory. To move a rat in or out of the cage it is necessary only to remove the screws at the ends of two or three rods and slide the rods through the openings.

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# TECHNIQUES FOR THE COLLECTION OF LYMPH FROM THE LIVER SMALL INTESTINE, OR THORACIC DUCT OF THE RAT

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WITH THE TECHNICAL ASSISTANCE OF EMERY VAN HOOK

THE following techniques for collection of lymph in the rat have been very satisfactory for periods of two or three days for lymph from the liver and for periods up to ten days for lymph from the intestine or from the thoracic duct. These methods have been useful in the study of lymph from the standpoint of the substances exchanged from the plasma to the lymph and of the materials contributed to the lymph from the intestine in the course of digestion and absorption of various foods.

Cannulation of the lymphatics of the liver is accomplished in rats weighing 150 to 300 grams. The rats are anesthetized with ether. Through a midline incision the liver is reflected upward to the right and the stomach and duodenum are reflected to the left, thus exposes the hepatogastric and hepatoduodenal ligaments. Evans blue dye, 0.1 ml. of a 0.5 per cent solution is injected through a fine hypodermic needle into the liver. Within a minute blue lymph may be seen in the lymphatics passing through the hepatogastric ligament. After the worker has acquired a little experience the clear lymphatics of the liver are easily recognized and the injection of dye is no longer necessary.

With the use of dissecting glasses, a blunt dissection with curved mosquito forceps proved the most practical method for isolation of the lymphatics. The inferior vena cava is dissected free above the right renal vein and a sharp 13 gauge needle is passed under the vena cava in line with the lymphatic vessel of the liver and through the abdominal wall. A plastic tube Transflex, 1 or 1.5 mm. in diameter with beveled ends, is filled with a dilute solution of heparin and passed through the needle to the outside. The needle is then removed. As much of the lymphatic as is exposed, usually 3 to 5 mm. is dissected free and ligated as far distally as possible. A small longitudinal opening is made in the lymphatic with the cutting edge of a 27 gauge hypodermic needle. One of the beveled tips of the plastic tubing is then passed into the lymphatic as far as possible and tied firmly in place (Fig. 1). A second ligature is placed near the vena cava so that the tubing is held in line with the direction of the lymphatic. Any accessory lymphatics in this region may be included in the ligature and since they anastomose freely, their flow is diverted to the cannulated lymphatic. Occasionally a small lymphatic from the duodenum may deliver cloudy lymph to the lymphatic vessel in the liver; this can be prevented by ligation of the small

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lymphatic connection In the rat which has been fed a diet containing fat, the lymph from the liver remains clear and contamination with intestinal lymph is easily recognized

The lymph begins to flow immediately through the tubes, and after the incision is repaired the animal is placed in the cage described in another paper<sup>1</sup> The lymph is collected in a graduated centrifuge tube from the plastic tube, the free end of which is passed through a small opening in a rubber cap on the centrifuge tube The plastic tube acts as a siphon and delivers the lymph best at 5 to 10 cm below the level of the rat

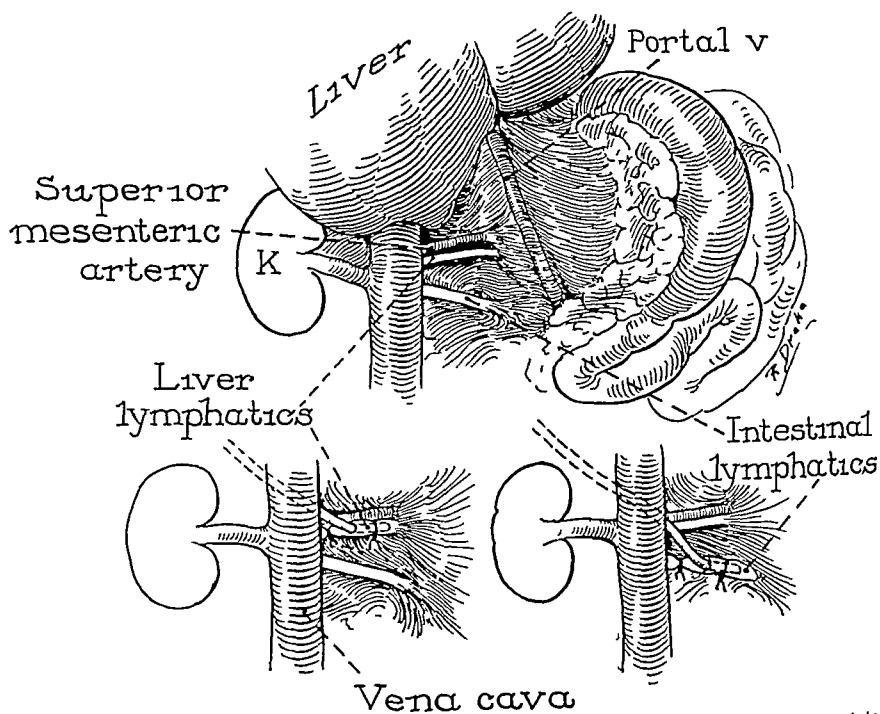


Fig 1—Structures involved in cannulation of a lymphatic of the liver and of an intestinal lymphatic.

Cannulation of the intestinal lymphatic which drains most of the small intestine is accomplished in a manner similar to that just described In the rat which has been fed a meal that contains fat the intestinal lymphatics are easily recognized because they appear in the lower portion of the hepatoduodenal ligament Evans blue dye injected into the substance of the intestine also stains the intestinal lymph blue There usually are accessory intestinal lymphatics which need to be ligated so that all the intestinal lymph may be collected through the main channel

Cannulation of the thoracic duct is accomplished in the etherized rat through an incision just distal to the last rib, extending from the midline anteriorly to the medial border of the left quadratus lumborum muscle posteriorly (Fig 2) A small gauze pack, placed in such a way that it pushes the stomach, liver, and intestines back and to the right, exposes the left portion of the dia

phragm, the aorta, and the left adrenal gland and kidney. A self containing mastoid retractor is used to retract the kidney distally and to hold the incision open. With the use of dissecting glasses a small opening is made in the peritoneum over the quadratus lumborum approximately 0.5 cm cephalad to the superior suprarenal artery. The veins and peritoneum are dissected superi-

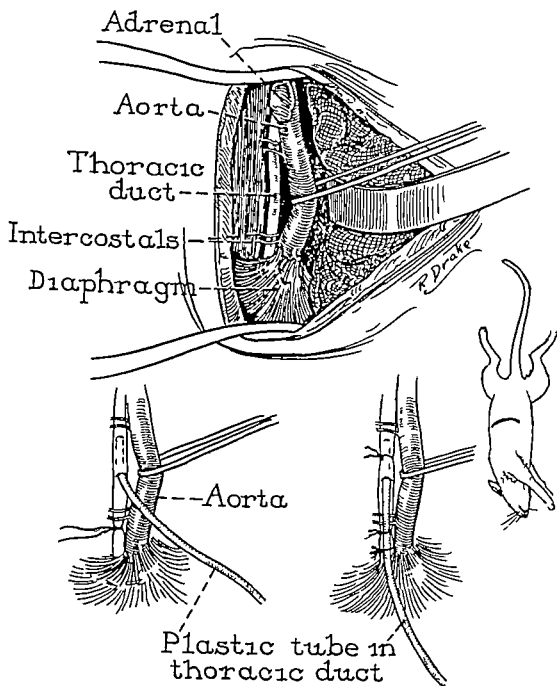


Fig. —Method of cannulation of the thoracic duct

cially and retracted to the right until the aorta is exposed. The aorta in this region is freed of attached tissue until the left subcostal artery is exposed. The thoracic duct is visible just posterior to the aorta. It is 1 to 2 mm in diameter and is embedded in loose connective tissue and fat. The thoracic duct is exposed for a length of 5 to 8 mm by gentle blunt dissection, and a ligature is passed around it at the upper end of the exposed portion just caudad to the subcostal artery. A second ligature is placed 3 to 5 mm caudad to the first. A sharp 13 gauge needle is passed through the abdomen at approximately the level of the xiphoid process and the beveled plastic tubes 1.5 mm in diameter, which contain

a dilute solution of heparin, are threaded through to the site of cannulation. A small longitudinal opening is made in the left anterior surface of the thoracic duct with the cutting edge of a 27 gauge hypodermic needle. This opening may be enlarged with a probe. A beveled end of the plastic tube is then slipped gently into the duct for 5 to 10 mm and tied firmly in place with the second ligature. The first ligature is tightened about the cannula and the ends of both ligatures are tied together for further security. The tube is arranged so that it will lie relatively straight in the duct and yet will curve along the diaphragm before it passes through the abdominal wall. The gauze pack is removed, the viscera are replaced in proper position, and the incision is closed.

After operation the rats are placed in cages constructed according to the description previously mentioned. These cages are small in order to prevent the rat from turning around, but they do permit some forward and backward movement. The volume of lymph obtained varies somewhat with the dietary and fluid balance of the animal. For normal rats which weigh 200 grams and receive a mixed diet and water as desired, approximately 5 cc of clear hepatic lymph are collected each twenty-four hours for two or three days, when dislodgment or clotting within the cannula terminates the flow of lymph. In similar rats under similar conditions approximately 20 cc of intestinal lymph are collected every twenty-four hours and the flow of lymph is usually continuous up to about ten days. From the thoracic duct approximately 25 cc of lymph are collected each twenty-four hours for periods up to ten or more days. This figure is slightly greater than that reported by Reinhardt<sup>2</sup> for nonfasting adult rats whose thoracic ducts were cannulated by a different approach while they were under the effects of sodium pentobarbital anesthesia.

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## STUDIES ON RH ANTIBODIES

### ANALYSIS OF A ZONE PHENOMENON IN AN RH ANTISERUM BY SPLITTING THE SERUM INTO TWO FRACTIONS BY MEANS OF DIALYSIS

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BUFFALO N Y

<sup>1</sup> For eighteen hours against large volumes of freshly distilled water, resulting in the division of serum into two fractions the precipitate and the supernatant, has been applied to the study of Rh antisera as reported in a previous communication<sup>1</sup>. The precipitate consisting mostly of globulins which are soluble in physiologic saline solution contains the major portion of the complete (saline) Rh agglutinins. The 'supernatant fraction' is a mixture composed of the albumin and a certain amount of the globulins. In this fraction are found anti Rh antibodies of the incomplete (albumin) variety. The method is of certain practical interest in that rather potent anti Rh testing reagents can be prepared from sera containing complete (saline) Rh agglutinins of such weak titer that they could not be used for diagnostic purposes. This is accomplished by dissolving the precipitate in a relatively small volume of saline solution. However, the agglutinating power of the 'globulin fraction' can be explained not only by concentration of the complete (saline) Rh agglutinins proper but also partly by their separation from the Rh antibodies of the incomplete (albumin) variety which tend to 'block' or suppress the saline agglutinins as long as the former also are present. Herein lies the theoretic interest in this procedure for it seems to prove that the two varieties of Rh antibodies occur in different serum fractions.

Since the original report was made a large number of additional anti Rh sera have been examined by this method or a slightly modified adaptation of it. Many sera have shown principally the same results when subjected to dialysis. Considering that the method is as crude as it is simple, it was to be expected that not all Rh antisera would follow the same pattern. One serum which was of special interest because it seemed to differ from the regular behavior is the subject of the investigations to be reported in this and the two following communications. It was obtained from an Rh negative patient who received multiple transfusions of whole blood, the majority of which most probably were Rh positive, stimulating the production of Rh antibodies. A brief case history of this patient follows below.

Mr. Rea had seven hospital admissions between February 1937 and his death in February, 1946. He suffered primarily from chronic ulcerative colitis which was treated initially by ileostomy and finally by colectomy. During his first six stays in the hospital he received a total of 4,000 cc of citrated, whole blood compatible as to the blood group but unknown

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The findings reported in this and the following communications were presented during a meeting in Washington, D. C. on Oct. 6 and 21, 1947, convened at the request of the Surgeon General of the United States Public Health Service for a discussion on the nomenclature of Rh typing sera.

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as to the Rh factor At the time of his final entrance into the hospital to undergo a total colectomy, he received in all 3,000 c c of citrated, whole blood belonging to his blood group but irrespective of the Rh type of the various bloods The last 500 c c produced a severe hemolytic transfusion reaction The analysis of the transfusion reaction carried out in our laboratory revealed the fact that the patient was an Rh negative individual whose serum contained an Rh antibody A large quantity of blood was collected from this patient immediately after his decease Examination of the serum obtained from this specimen showed an Rh antibody of the complete (saline) variety of only weak titer but one of the (albumin) variety of high titer However, in no dilution of the patient's serum, agglutination of Rh positive cells greater than 2 plus macroscopically in the initial investigations carried out The relatively weak degree of agglutination excluded the use of this serum for practical purposes as a diagnostic reagent Accordingly, it was decided to dialyze a sample of it

Two fractions were obtained by dialyzing 100 c c amounts of this serum for eighteen hours against two changes of 12 liters each of freshly distilled water in the refrigerator at approximately 4° C The precipitate, to be referred to as the "globulin fraction," was separated by centrifugation from the remaining dialysate, to be referred to as the "supernatant fraction" The "globulin fraction" was dissolved in 10 c c of 0.9 per cent saline solution corresponding to one tenth the amount of the original serum specimen (a ten times concentration) and lyophilized in small ampules containing 10 c c each When needed, they were dissolved by the addition of 10 c c of distilled water The salt free "supernatant fraction" was also lyophilized and when needed was dissolved in physiologic saline solution The experiments recorded in this paper all refer to the two serum fractions obtained in this manner

In order to compare the relative distribution of the complete (saline) and incomplete (albumin) Rh antibodies in the two serum fractions with that in the native (untreated) serum, the first experiment was carried out in two parts In Part I saline was used as the medium for preparing the dilutions of the serum and its fractions and for suspending the Rh positive test cells, while in Part II, undiluted, normal, adult, human serum of Group O was used as a diluent Experiment I itself was carried out in the following manner

Decreasing amounts of native serum (Ree) and its two fractions, volume 0.05 c c, were mixed with 0.05 c c of a 2 per cent suspension of homozygous Rh<sub>i</sub> (CDe/Ce) cells belonging to blood Group O The tubes were shaken thoroughly After standing for one hour at room temperature they were centrifuged at approximately 1,500 revolutions per minute for two minutes and read macroscopically for agglutination as recorded in Table I

This experiment shows that the complete (saline) Rh antibodies, which were very weak from the start, are somewhat concentrated in the "globulin fraction" corresponding to previous experiences The "supernatant fraction" does not contain any complete (saline) anti-Rh agglutinins When saline is replaced as a diluent by undiluted, normal, adult, human serum, incomplete (albumin) Rh agglutinins of considerable titer become apparent in the native serum In this experiment, however, the degree of agglutination of Rh-positive test cells does not exceed the 2-plus stage in any of the dilutions tested A zone phenomenon in the middle of the titration can be recognized In contrast to the native serum, the "globulin fraction" produces a 4-plus agglutination of the Rh positive cells which is stronger than might be expected even if it is realized that this fraction corresponds to a ten times concentration of the original native serum This "globulin fraction" also differs from "globulin fractions" previously described inasmuch as it contains Rh antibodies of the incomplete (albumin) variety reacting much stronger if undiluted serum is used as a diluent instead of saline solution

The second fraction, "the supernatant," reveals a prozone phenomenon which is absent both in the native serum and in the "globulin fraction" On

TABLE I AGGLUTINATION OF RH POSITIVE GROUP O CELLS BY SERUM (REE) AND ITS TWO FRACTIONS ( GLOBULIN AND SUPERNATANT )

DILUENT	PART I 0.9% SALINE			PART II UNDILUTED HUMAN SERUM		
	A NATIVE	B GLOBULIN	C SUPERNATANT	A NATIVE	B GLOBULIN	C SUPERNATANT
SERUM (REE)						
1 Undiluted	+	++	-	++	++++	-
2 1 2	±	+	-	++	++++	±
3 1 4	-	+	-	++	++++	±
4 1 8	-	±	-	+	++++	+
5 1 16	-	±	-	+	++++	++
6 1 32	-	±	-	+	++++	++
7 1 64	-	+	-	++	+++	+
8 1 128	-	+	-	++	++	+
9 1 256	-	±	-	++	++	++
10 1 512	-	-	-	++	+	++
11 1 1024	-	-	-	++	-	++
12 0	-	-	-	-	-	-

- No agglutination ± Faint agglutination + Slight agglutination ++ Marked agglutination +++ Strong agglutination ++++ Very strong agglutination

could readily visualize how the combined action of the two separate serum fractions could result in the type of agglutination that is shown by the native serum

Inasmuch as the experiment shown in Table I did not reach the end point of agglutination and because of the zone phenomenon observed a second experiment was carried out as follows

Decreasing amounts of the native serum (Ree) and its two fractions respectively, volume 0.05 cc were mixed with 0.05 cc of a 2 per cent suspension of homozygous Rh (CDe/Ce) Group O cells. The mixtures were allowed to remain for one hour at room temperature and then were spun down. The resulting agglutinations as read macroscopically are shown in Table II

TABLE II AGGLUTINATION OF RH POSITIVE GROUP O CELLS BY THE INCOMPLETE ANTI RH ANTIBODIES OF SERUM (REE) AND OF ITS TWO FRACTIONS—END POINT TITRATIONS

SERUM (REE)	A NATIVE	B GLOBULIN	C SUPERNATANT
1 Undiluted	+++	++++	-
2 1 2	++	++++	-
3 1 4	++	++++	±
4 1 8	++	++++	+
5 1 16	+	++++	++
6 1 32	+	++++	++
7 1 64	++	++++	+++
8 1 128	++	+++	+++
9 1 256	+++	+++	+++
10 1 512	+++	+++	+++
11 1 1024	+++	+++	++
12 1 2048	+++	+++	++
13 1 4096	+++	+++	++
14 1 8192	++	++	+
15 1 16384	++	++	±
16 1 32768	++	++	-
17 1 65536	+	++	-
18 1 131072	+	+	-
19 1 262144	+	+	-
20 1 524288	±	+	-
21 1 1048576	-	±	-
22 1 2097152	-	-	-
23 1 4194304	-	-	-
24 0	-	-	-

All dilutions made with undiluted normal adult human serum of Group O

Experiment II again demonstrates the fact that the native serum upon titration exhibits a zone phenomenon most obviously in dilutions of 1:16 or 1:32, reaching an end point titer of approximately 260,000\*. The "globulin fraction" reveals the presence of an incomplete (albumin) variety of Rh antibody of considerable strength decreasing in potency in a straight line with an end point of approximately 1:500,000\* without showing any zone phenomenon. No agglutination occurs in the undiluted "supernatant fraction." As this fraction is diluted further an incomplete (albumin) anti-Rh antibody of increasing strength becomes apparent until a dilution of 1:512 is reached, whereafter it decreases, reaching an end point titer of approximately 1:8,000\*.

The preceding experiments focused attention on the peculiar behavior of the "supernatant fraction" when titrated in undiluted, normal, adult, human serum. Was the prozone phenomenon due to surplus inhibition or to the activity of an antibody truly blocking in nature? When Wiener<sup>2</sup> first described his Rh "blocking antibody" ("incomplete" Rh antibody of Race<sup>3</sup>), he demonstrated its presence by its "blocking" effect on the Rh saline agglutinin (complete Rh antibody). However, experiments by Diamond and associates<sup>4,5</sup> and Wiener<sup>1</sup> revealed that the so-called "blocking" antibody did not "block" at all if as a diluent saline solution were replaced by 20 per cent bovine albumin solution or undiluted human serum. Use of these latter substances as diluents resulted in agglutination of Rh-positive cells treated with such a "blocking" or "incomplete" antibody. In the case under discussion, undiluted, normal, adult, human serum was used as a diluent and still a prozone phenomenon was observed.

The blocking effect of the "supernatant fraction" was tested in Experiment III following a similar order of experiment as used in an earlier communication<sup>1</sup>. The cell suspension and the dilutions of the "supernatant fraction" were all made in undiluted human serum instead of in physiologic saline solution. The experiment itself was carried out thusly:

Decreasing amounts of (A) "the supernatant fraction" of serum (Ree) and (B) the "supernatant fraction" of a normal, adult serum, used in this experiment as a control, volume 0.15 cc., were mixed with 0.05 cc. of a 6 per cent suspension of homozygous Rh<sub>1</sub> (CD<sub>5</sub>/Ce) cells belonging to blood Group O. The experiment was set up in duplicate (Part I and Part II). After incubation for one hour at room temperature, the following anti-Rh sera were added to each tube in Part I, 0.05 cc. of undiluted "globulin fraction" of serum (Ree) to each tube in Part II, 0.05 cc. of undiluted anti-D (Rh<sub>0</sub>) serum (Dad) containing an Rh antibody of the incomplete (albumin) variety. The tubes after being shaken well were kept for an additional hour at room temperature, then centrifuged and read macroscopically for agglutination. The results obtained are recorded in Table III.

It can be seen that the "supernatant fraction" of serum (Ree) does indeed inhibit or block its own "globulin fraction," thus preventing the "globulin fraction" from agglutinating the Rh-positive test cells. It also prevents the agglutination of Rh-positive cells by anti-Rh agglutinins of the incomplete (albumin) variety contained in serum (Dad) (Part II) as compared with the "supernatant fraction" of the normal serum used as a control.

In Experiment III, decreasing amounts of the "supernatant fraction" showing the blocking phenomenon were mixed with constant amounts of the "globulin

\*Inasmuch as pipettes were not changed with each dilution these figures should not be taken as absolute values.



TABLE III. INHIBITORY EFFECT OF THE 'SUPERNATANT FRACTION' OF SERUM (REE) ON THE AGGLUTINATION OF RH POSITIVE GROUP O CELLS BY RH AGGLUTININS OF THE INCOMPLETE VARIETY

SUPERNATANT FRACTIONS	PART I GLOBULIN FRACTION (REE)		PART II ANTI RH SERUM (DAD)	
	A SERUM (REE)	B NORMAL SERUM	A SERUM (REE)	B NORMAL SERUM
1 Undiluted	±	+++	-	+++
2 1 3	+	++++	±	++++
3 1 9	++	++++	+	++++
4 1 27	++	++++	++	++++
5 1 81	++	++++	++	++++
6 0	++++	++++	++++	++++

All dilutions made with undiluted normal adult human serum of Group O

fraction' and with another anti Rh serum containing incomplete (albumin) Rh antibodies. The next experiment to be reported reveals the blocking effect of this "supernatant fraction" of serum (Ree) when it is added in a constant amount to decreasing amounts of its own globulin fraction' and its own supernatant fraction". The experiment was performed as follows

Decreasing amounts of (A) the "globulin fraction" obtained from anti Rh serum (Ree) and (B) the "supernatant fraction" obtained from anti Rh serum (Ree) volume 0.05 cc, were mixed in Part I with 0.1 cc of undiluted supernatant fraction of serum (Ree), and in Part II with 0.1 cc of undiluted "supernatant fraction" of a normal serum. After the tubes were shaken, 0.05 cc of a 5 per cent suspension of Rh (cDE/c) Group O cells was added to each. The tubes after remaining one hour at room temperature were centrifuged at 1500 revolutions per minute for two minutes; the resulting agglutinations were read macroscopically.

TABLE IV. AGGLUTINATION OF RH POSITIVE GROUP O CELLS BY THE GLOBULIN AND SUPERNATANT FRACTIONS OF SERUM (REE) AFTER BEING MIXED WITH THE 'SUPERNATANT FRACTION' OF SERUM (REE)

FRACTIONS OF ANTI RH SERUM (REE)	PART I SUPERNATANT FRACTION OF SERUM (REF)		PART II SUPERNATANT FRACTION OF NORMAL SERUM	
	A GLOBULIN	B SUPERNATANT	A GLOBULIN	B SUPERNATANT
1 Undiluted	+++	-	++++	-
2 1 2	+++	-	++++	-
3 1 4	++	-	++++	±
4 1 8	++	-	++++	+
5 1 16	+	-	++++	+
6 1 32	±	-	+++	++
7 1 64	-	-	+++	++
8 1 128	-	-	+++	+++
9 1 256	-	-	+++	++
10 1 512	-	-	++	+
11 1 1024	-	-	++	+
12 1 2048	-	-	++	±
13 1 4096	-	-	++	-
14 1 8192	-	-	++	-
15 1 16384	-	-	+	-
16 1 32768	-	-	+	-
17 1 65536	-	-	±	-
18 1 131072	-	-	±	-
19 1 262144	-	-	-	-
20 1 524288	-	-	-	-
21 1 1048576	-	-	-	-
22 1 2097152	-	-	-	-
23 0	-	-	-	-
24 0 + 0.1 cc serum diluent	-	-	-	-

All dilutions made with undiluted normal adult human serum of Group O

and recorded in Table IV. In this experiment all dilutions and cell suspensions were prepared with undiluted, normal, adult, human serum of Group O.

When a constant amount of the undiluted "supernatant fraction" of Rh antiserum (Ree) is added to decreasing amounts of its own "globulin fraction," a marked suppression of the agglutination of the Rh-positive test cells results, reducing the titer of its own "globulin fraction" from 32,000 to 16. Furthermore, the undiluted "supernatant fraction" of serum (Ree) when added in constant amount to decreasing amounts of the same "supernatant fraction" of serum (Ree) itself, completely suppresses and prevents the agglutination of the Rh-positive cells by the incomplete (albumin) Rh antibody contained in the greater dilutions of this "supernatant fraction." In contrast, the addition of the "supernatant fraction" of a normal serum fails to prevent the agglutination of the Rh-positive test cells by the "globulin fraction" and the "supernatant fraction" of serum (Ree).

In the experiments described thus far, the blocking effect of the "supernatant fraction" of the anti-Rh serum (Ree) was tested against its own "globulin fraction," which contains a very potent incomplete (albumin) Rh antibody, and against one additional anti-Rh serum (Dad) containing the same type of Rh antibodies but of lower titer. What effect would the "supernatant fraction" of serum (Ree) have on anti-Rh antibodies of the complete (saline) variety? To find the answer to this question, two anti-Rh sera with antibodies of the complete (saline) variety and an additional anti-Rh serum with antibodies of the incomplete (albumin) variety were tested in Experiment V. All of the dilutions and the cell suspension were made with undiluted, normal, adult, human serum of Group O. This experiment was carried out in the following manner:

Decreasing amounts of (A) anti Rh globulin (Dib), containing Rh antibodies of the complete (saline) variety, (B) anti Rh serum (And), containing Rh antibodies of the complete (saline) variety, and (C) anti Rh serum (Dad), containing Rh antibodies of the incomplete (albumin) variety, volume 0.05 cc, were mixed in Part I with 0.1 cc of undiluted "supernatant fraction" of serum (Ree) and in Part II with 0.1 cc of undiluted "supernatant fraction" of a normal serum. Each tube received 0.05 cc of a 5 per cent suspension of Rh (cDE/c) cells belonging to the blood Group O. Following the addition of the test cells the mixtures were incubated for one hour at room temperature and were then centrifuged. The resulting agglutinations are recorded in Table V.

TABLE V. BLOCKING EFFECT OF "SUPERNATANT FRACTION" OF SERUM (REE) ON AGGLUTINATION OF RH POSITIVE GROUP O CELLS BY THREE ANTI RH SERA

ANTI RH SERA	PART I SUPERNATANT FRACTION OF SERUM (REE)			PART II SUPERNATANT FRACTION OF NORMAL SERUM		
	A GLOBULIN (DIB)	B SERUM (AND)	C SERUM (DAD)	A GLOBULIN (DIB)	B SERUM (AND)	C SERUM (DAD)
1 Undiluted	—	—	—	++++	+++	++
2 1:2	—	—	—	++++	+++	+
3 1:4	—	—	—	+++	++	—
4 1:8	—	—	—	+++	+	—
5 1:16	—	—	—	++	+	—
6 1:32	—	—	—	+	—	—
7 1:64	—	—	—	+	—	—
8 1:128	—	—	—	+	—	—
9 1:256	—	—	—	—	—	—
10 0	—	—	—	—	—	—

- A Globulin (Dib) contains anti-Rh antibodies of the incomplete (albumin) variety  
 B Serum (And) contains anti-Rh antibodies of the complete (saline) variety  
 C Serum (Dad) contains anti-Rh antibodies of the incomplete (albumin) variety

It can be seen from this experiment that the 'supernatant fraction' of serum (Ree) completely suppresses the agglutination of Rh positive cells by anti Rh agglutinins irrespective of whether they are of the incomplete or the complete variety. The supernatant fraction prepared from a normal human serum does not prevent the agglutination of the Rh positive cells by any of the anti Rh sera tested.

#### DISCUSSION

Zone phenomena frequently have been seen upon quantitative titration of Rh antisera. Taylor, Race, Pinner, and Ikin<sup>10</sup> reported an anti Rh containing serum which when used undiluted caused according to their interpretation, the agglutination of cells classed as strongly positive reactors to Rh antibodies but failed to agglutinate weaker cells. Upon serial dilutions of this serum these weakly reacting cells also were agglutinated. The authors suggested at this time that the titration method should be applied to sera for the detection of Rh antibodies.

Attention was called by Levine<sup>11</sup> to the fact that prozone phenomena were occasionally observed in anti Rh sera. He warned that such sera should not be used for routine Rh typing. The same author with Waller<sup>1</sup> in additional experiments carried out on sera exhibiting prozones reported that this phenomenon could be explained by the fact that such sera contained a mixture of complete (saline) and incomplete (albumin) Rh agglutinins. The latter antibody acted as a 'blocking antibody' preventing the agglutination of Rh positive cells in the first few dilutions. By absorption with Rh positive cells this prozone could be eliminated, apparently due to the removal of the incomplete (albumin) type of Rh antibody.

After the investigations of various authors had shown that the 'blocking' antibody of Wiener, or the incomplete antibody of Race could be demonstrated directly when undiluted human serum or 20 per cent bovine albumin replaced saline solution as the diluent. Levine<sup>12</sup> reported that quantitative studies on the 'direct reaction' of this so called 'blocking' antibody revealed the presence of a prozone phenomenon in certain instances. He raised the question whether there were not two varieties of 'blocking' antibodies.

Hattersley and Fawcett<sup>14</sup> reported three instances of Rh antisera showing prozones when titrated in undiluted human serum and tested against Rh positive cells suspended in 30 per cent bovine albumin. The prozones observed by them did not appear at room temperature but only following incubation of the serum cell mixtures for one hour at 37° C. This prozone occurred only with cells of the subtype Rh<sub>0</sub> but not with Rh' or Rh'' cells. If the tubes were centrifuged immediately after the titration was completed the prozone phenomenon failed to appear. As a result of this observation Hattersley<sup>15</sup> suggested a modification of the technique of routine Rh typing using the readily available Rh antisera containing Rh agglutinins of the incomplete (albumin) variety and centrifuging at high speed without preliminary incubation in order to eliminate falsely negative reactions as a result of the prozone phenomenon. It is interesting to note that the prozone phenomenon increased in Hattersley and Fawcett's case 3 following stimulation of the patient with small injections of Rh positive blood belonging to the subtype Rh<sub>1</sub>.

## SUMMARY AND CONCLUSIONS

The method of splitting anti-Rh sera into two fractions by dialysis, namely the precipitate (globulins) and the supernatant (mixture of albumin and globulins), has been applied to the serum of an Rh-negative patient (Ree) who produced Rh antibodies following multiple transfusions of Rh-positive blood over a period of several years. The comparison of the untreated serum with its two fractions led to the following observations:

1 The native, untreated serum when tested for Rh antibodies, using undiluted human serum as a diluent, contained Rh agglutinins of the incomplete (albumin) variety up to a titer of approximately 260,000. However, definite zones of agglutination became apparent upon quantitative titration.

2 Marked agglutination of Rh-positive cells decreasing in strength in a straight line upon dilution was produced by the "globulin fraction." No zone phenomenon was observed with this fraction.

3 The second fraction, the "supernatant," showed a strong prozone phenomenon in the first few dilutions. By "combining" the two fractions, the "globulin" and the "supernatant," the irregular zone phenomenon of the native, untreated serum can be understood.

4 The "supernatant fraction" of serum (Ree) blocks the agglutination of Rh-positive cells by both the complete (saline) and the incomplete (albumin) varieties of Rh antibodies. This is in contrast to the blocking effect of the Rh incomplete (albumin) antibody which inhibited the agglutination of Rh-positive cells by the complete (saline) Rh antibody only but induced agglutination of Rh-positive cells by its own action when saline solution was replaced by undiluted normal serum or albumin solution.

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## STUDIES ON RH ANTIBODIES

### II THE DEMONSTRATION OF A THIRD TYPE OF RH ANTIBODY WITH BLOCKING PROPERTIES

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THE preceding communication<sup>1</sup> described experiments carried out on an anti Rh serum obtained from an Rh negative patient who had received multiple transfusions of Rh positive blood. This serum showed a peculiar zone phenomenon upon quantitative titration. When divided into two fractions by dialysis, the precipitate (the globulin fraction) and the supernatant (the "supernatant fraction"), the globulin fraction produced agglutination of Rh positive cells decreasing in potency in a straight line following the serial dilutions without exhibiting a zone phenomenon. In contrast the supernatant fraction showed a definite prozone phenomenon which was not evident in the native serum itself.

Prozone phenomena have been encountered frequently during Rh antibody investigations as mentioned in the preceding paper.<sup>1</sup> The experiments to be reported in this second communication deal with the problem of whether the prozone phenomenon found in the "supernatant fraction" of serum (Ree) is due to a surplus inhibition (surplus of Rh antibodies) or is caused by the presence of a peculiar antibody exhibiting a blocking effect which in contrast to the "blocking" antibody described by Wiener,<sup>2</sup> manifests itself even when undiluted, normal, adult, human serum is used as a diluent.

Assuming that we are dealing with a true antibody effect and not a surplus inhibition, Rh positive cells treated with such an antibody should remain blocked although subjected to multiple washings with physiologic saline solution or undiluted normal, human serum. On the other hand if the zone phenomenon were due to a surplus of Rh antibodies multiple washings of the sensitized cells should result in a removal of this surplus of antibodies thus causing agglutination to occur. The experiments to be reported herein deal with this problem. From the technical point of view two different procedures were involved in each experiment: one, in which the Rh positive cells were treated with the "supernatant fraction" of serum (Ree) followed by a series of thorough washings of these blood cells, and the second, which consisted of the actual testing of the sensitized cells by means of the addition of anti Rh sera containing different varieties of Rh antibodies.

The first experiment well illustrates the principal procedures employed. It was performed in order to determine whether or not the supernatant frac-

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tion" of serum (Ree) contained an antibody which would sensitize Rh positive cells and thus prevent their agglutination when subsequently mixed with anti Rh serum. Experiment I was carried out as follows:

#### I Method of Sensitization of Rh Positive Cells—

- 1 0.5 cc of a 10 per cent suspension of Rh<sub>+</sub> (cDE/c) cells belonging to blood Group O was added to each of ten tubes in two rows of five tubes each.
- 2 To Row (A), increasing dilutions (1) undiluted, (2) 1:3, (3) 1:9, (4) 1:27, and (5) 1:81 of the "supernatant fraction" of serum (Ree) were added in a volume of 1.5 cc per tube. A total volume of 2.0 cc was obtained in all tubes, the only difference being the amount of the "supernatant fraction" present in each tube.
- 3 The tubes were shaken well and kept for thirty minutes in the refrigerator at 4° C.
- 4 After centrifuging the tubes for ten minutes at 4,500 revolutions per minute, the supernatant fluids were aspirated and discarded.
- 5 The sediments, consisting of packed red blood cells, were washed thoroughly three times using 5.0 cc of ice cold 0.9 per cent saline solution per tube.\*
- 6 After the third washing all of the saline solution was removed as completely as possible by aspiration.
- 7 0.5 cc of undiluted, normal, adult, human serum was added to each tube and the sedimented cells were resuspended completely in the serum.
- 8 A parallel Row (B) was set up under identical experimental conditions (steps 1-7), the only difference being that the "supernatant fraction" of a normal serum was used (control).

II Method of Testing the Sensitized Rh Positive Cells for Agglutination With Anti Rh Sera—0.05 cc of the cell suspensions was removed from each of the tubes of Row (A) and Row (B) and pipetted into a new series of small tubes. The experiment was carried out in duplicate. To each tube in Part I, 0.05 cc of undiluted anti D (Rh<sub>0</sub>) serum containing Rh agglutinins of the complete (saline) variety was added, and to each tube in Part II, 0.05 cc of undiluted anti D (Rh<sub>0</sub>) serum containing Rh agglutinins of the incomplete (albumin) variety was added. After being allowed to stand for thirty minutes at room temperature, the tubes were spun down for two minutes at 1,500 revolutions per minute. The resulting agglutinations are seen in Table I.

TABLE I AGGLUTINATION BY ANTI RH SERA OF RH POSITIVE CELLS TREATED WITH THE "SUPERNATANT FRACTION" OF SERUM (REE)

SUPERNATANT FRACTIONS	PART I ANTI D (RH <sub>0</sub> ) AGGLUTININS COMPLETE (SALINE) VARIETY		PART II ANTI D (RH <sub>0</sub> ) AGGLUTININS INCOMPLETE (ALBUMIN) VARIETY	
	A SERUM (REE)	B NORMAL SERUM	A SERUM (REE)	B NORMAL SERUM
1 Undiluted	—	++++	—	++++
2 1:3	—	++++	+	+++
3 1:9	—	++++	+	++
4 1:27	+	++++	++	++
5 1:81	++	++++	+++	+++

All dilutions made with undiluted normal adult human serum of Group O

- No agglutination
- ± Faint agglutination
- + Slight agglutination
- ++ Marked agglutination
- +++ Strong agglutination
- ++++ Very strong agglutination

\*Physiologic saline solution was used for all of the dilutions and washing as previous experiments revealed that there was no difference in the outcome of the experiments irrespective of whether saline or undiluted normal adult human serum was used.

According to Experiment I Rh positive cells treated with the supernatant fraction of serum (Rec) fail to be agglutinated upon the addition of Rh antibodies of both the complete (saline) and the incomplete (albumin) variety depending upon the amount of the supernatant fraction of the serum (Rec) used for the sensitization of these cells. The complete (saline) type of Rh agglutinin seems to be blocked more easily than the incomplete (albumin) type. In contrast, the 'supernatant fraction' obtained from a normal adult human serum does not exhibit any blocking properties indicating the specificity of the effect observed.

The results of this experiment were corroborated in many others carried out in an identical or similar manner and seem to suggest that the prozone phenomenon observed in the supernatant fraction of serum (Rec) is due to a peculiar type of Rh antibody rather than to the presence of a surplus of Rh antibodies which should have been removed by the multiple washings.

The question arose whether the inhibition of agglutination shown in the first experiment could be reproduced with an Rh antibody of the known incomplete (albumin) variety previously described by Rice<sup>3</sup> and Wiener or whether the blocking phenomenon was caused by a different third type of Rh antibody present in the 'supernatant fraction' of serum (Rec).

In an attempt to answer this problem an experiment was carried out on a qualitative basis comparing the supernatant fraction of serum (Rec) with the 'supernatant fraction' of anti Rh serum (Fis) known to contain Rh agglutinins of the incomplete (albumin) variety. Experiment II was performed as follows:

#### I. Method of Sensitization of Rh Positive Cells —

- 1 0.5 cc of a 10 per cent suspension of heterozygous Rh (CDe/ce) cells belonging to blood Group O was added to each of three tubes.
- 2 0.5 cc of undiluted supernatant fractions was added to each tube as follows:  
 Tube (A) 'Supernatant fraction' of serum (Rec)  
 Tube (B) 'Supernatant fraction' of serum (Fis)  
 Tube (C) Supernatant fraction of a normal adult human serum (control)
- 3 The three tubes were shaken well and kept for thirty minutes in the refrigerator at 4°C. Following this incubation period examination of the tubes revealed agglutination in tube (B) with the supernatant fraction of serum (Fis) known to contain incomplete (albumin) Rh agglutinins. No visible agglutination was detectable in the other two tubes (A) and (C).
- 4 The tubes were centrifuged for ten minutes at 4500 revolutions per minute and the supernatants were aspirated and saved for further testing as will be described in a later experiment (Experiment III).
- 5 The cell sediments were washed thoroughly three times using 5.0 cc of ice cold physiologic saline solution for each tube. When saline solution was added the first time the packed cells in tubes (A) and (C) completely dispersed showing no agglutination. However the cells in tube (B) which had been treated with the incomplete (albumin) Rh agglutinins of the 'supernatant fraction' of serum (Fis), again showed definite agglutination. This clumping was completely broken up by vigorous shaking and inverting of the tube. Some agglutination still occurred in tube (B) following the second washing but to a much lesser degree than at the time of the first washing. When the cells were washed a third time no visible agglutination was noticeable.

- 6 After the third washing and centrifugation the saline solution was removed as thoroughly as possible by aspiration
- 7 0.5 cc of undiluted, normal, adult, human serum then was added to each of the three tubes and the packed, sedimented cells were resuspended completely

**II Method of Testing the Sensitized Rh-Positive Cells for Agglutination With Anti Rh Sera**—To 0.05 cc of each of the three sensitized cell suspensions pipetted into three rows of three small tubes each were added in Row (1), 0.05 cc undiluted anti D ( $Rh_0$ ) serum containing Rh agglutinins of the complete (saline) variety, in Row (2), 0.05 cc undiluted anti D ( $Rh_0$ ) serum containing Rh agglutinins of the incomplete (albumin) variety, and in Row (3), 0.05 cc of undiluted, normal, adult, human serum. After standing for one hour at room temperature, the tubes were centrifuged for two minutes at 1,500 revolutions per minute and read macroscopically for agglutination. The results are seen in Table II.

TABLE II AGGLUTINATION BY ANTI RH SERA OF RH POSITIVE CELLS TREATED WITH THE "SUPERNATANT FRACTIONS" OF SERUM (REE), SERUM (FIS), AND A NORMAL SERUM

	SUPERNATANT FRACTIONS		
	A SERUM (REE)	B SERUM (FIS)	C NORMAL SERUM
1 Anti D ( $Rh_0$ ) agglutinins (complete)	—	++++	++++
2 Anti D ( $Rh_0$ ) agglutinins (incomplete)	—	++++	++++
3 Normal, adult, human serum	—	±	—

All dilutions made with undiluted, normal adult human serum of Group O

The "supernatant fraction" of serum (Ree) completely suppresses agglutination of the test cells. In contrast, the "supernatant fraction" of a serum containing Rh antibodies of the incomplete (albumin) variety fails to inhibit the agglutination of Rh-positive cells upon the subsequent addition of Rh agglutinins of either the complete (saline) or the incomplete (albumin) variety, behaving exactly as the "supernatant fraction" of the normal serum.

Attention should be called to the fact that Rh-positive cells treated with a "supernatant fraction" containing incomplete (albumin) Rh antibodies were only slightly agglutinated, if at all, following their resuspension in undiluted, normal, adult, human serum (3B). This observation suggests that the incomplete (albumin) type of Rh agglutinins can be removed from Rh positive cells by simple washing, a finding which will be discussed later in detail.

As mentioned, the supernatants obtained in step 4 during the preparation of the preceding experiment were kept for further study. They are nothing else but the absorbed "supernatant fractions" of the Rh antisera used in Experiment II. It was of interest to find out in what way the Rh agglutinin content of these sera was affected by treatment with Rh-positive cells.

Experiment III shows the influence of absorption upon the Rh antibody content of the "supernatant fraction" of the two anti-Rh sera. The titration were set up in two parts as follows:

Decreasing amounts of the "supernatant fractions" of sera (Ree) and (Fis) respectively, volume 0.05 cc, in Part I, before absorption, and in Part II, after absorption, were mixed each with 0.05 cc of a 2 per cent suspension of heterozygous  $Rh_1$  ( $CD_{45/45}$ ) Group O cells. The tubes were shaken thoroughly and allowed to stand for forty-five minutes at room temperature. They then were centrifuged and read macroscopically for agglutination as recorded in Table III. As a diluent for all the dilutions and the test cell suspension, undiluted, normal, adult, human serum of Group O was used.



TABLE III AGGLUTINATION OF RH POSITIVE CELLS BY THE ' SUPERNATANT FRACTIONS ' OF SERUM (REE) AND SERUM (FIS) BEFORE AND AFTER ABSORPTION

SUPERNATANT FRACTION	PART I BEFORE ABSORPTION		PART II AFTER ABSORPTION	
	A SERUM (REE)	B SERUM (FIS)	C SERUM (REE)	D SERUM (FIS)
1 Undiluted	-	++++	-	+
2 1 2	-	++++	-	±
3 1 4	+	++++	+	±
4 1 8	+	+++	++	-
5 1 16	++	++	++++	-
6 1 32	++	+	++++	-
7 1 64	+++	±	++++	-
8 1 128	+++	-	++++	-
9 1 256	+++	-	++++	-
10 1 512	++	-	++++	-
11 1 1,024	++	-	+++	-
12 0	-	-	-	-

All dilutions made with undiluted normal adult human serum of Group O

The comparison of the supernatant fractions of serum (Ree) and serum (Fis) respectively reveals an interesting contrast. Treatment of the supernatant fraction of serum (Ree) with Rh positive cells has resulted in a surprising increase in the degree of agglutination obtained rather than in a decrease as would be expected. On the other hand treatment of the supernatant fraction of serum (Fis) carried out under identical conditions has resulted in a considerable loss of the Rh antibody content. Apparently a blocking type of Rh antibody has been absorbed from the supernatant fraction of serum (Ree) allowing the incomplete (albumin) type of Rh antibody also present to act on the Rh positive test cells to a greater degree than before absorption. This paradoxical phenomenon seems to be explained best by the assumption of differences in the avidity of the various types of Rh antibodies for Rh positive cells.

That absorption of the supernatant fraction of serum (Ree) with Rh positive cells indeed increases rather than decreases its agglutinating potency under certain quantitative conditions is shown in the following Experiment IV

#### I Method of Absorption of Supernatant Fraction of Serum (Ree)\*—

- 1 10 cc of the undiluted "supernatant fraction" of serum (Ree) was added to 10 cc of three washed packed heterozygous Rh (CDe/cc) Group O cells, mixed and kept for thirty minutes in the refrigerator at 4°C.
- 2 Following centrifugation for ten minutes at 4500 revolutions per minute the supernatant fluid was aspirated, saved and labelled "Absorbed Fluid No 1". An aliquot was removed for testing purposes.
- 3 The remaining "Absorbed Fluid No 1" was reabsorbed with an equal volume of washed packed cells kept at 4°C and centrifuged; the supernatant was aspirated and labelled "Absorbed Fluid No 2".

II Quantitative Testing of the Absorbed Supernatant Fraction of Serum (Ree)—Decreasing amounts of (a) the supernatant fraction of serum (Ree) before absorption (b) after the first absorption ("Absorbed Fluid No 1") (c) after the second absorption ("Absorbed Fluid No 2") volume 0.05 cc were mixed with 0.05 cc of a 2 per cent suspension of heterozygous Rh (CDe/cc) Group O cells. Undiluted human serum was used as a

For this experiment the lyophilized supernatant fraction of serum (Ree) was dissolved in undiluted normal adult human serum of Group A instead of in 0.9 per cent saline solution.

diluent throughout the experiment. After standing for forty five minutes at room temperature the tubes were centrifuged at 1,500 revolutions per minute for two minutes. The resulting agglutination as read macroscopically is recorded in Table IV.

TABLE IV AGGUTINATION OF RH POSITIVE CELLS BY THE "SUPERNATANT FRACTION" OF SERUM (REE) AND THE ABSORBED FLUIDS PREPARED FROM THIS FRACTION

SUPERNATANT FRACTION OF SERUM (REE)	A UNTREATED	B ABSORBED FLUID NO 1	C ABSORBED FLUID NO 2
1 Undiluted	-	±	++
2 1 2	-	+	++
3 1 4	+	++	+
4 1 8	+	++	+
5 1 16	++	+++	++
6 1 32	++	+++	+++
7 1 64	++	++++	++
8 1 128	+++	++++	++
9 1 256	+++	++++	+
10 1 512	++	++++	±
11 1 1,024	++	+++	-
12 1 2,048	±	++	-
13 1 4,096	±	+	-
14 1 8,192	-	±	-
15 1 16,384	-	±	-
16 1 32,768	-	-	-
17 1 65,536	-	-	-
18 0	-	-	-

All dilutions made with undiluted normal adult human serum of Group O

Treatment of the "supernatant fraction" of serum (Ree) with an equal volume of packed Rh-positive cells increases the degree of agglutination of Rh positive cells produced by this fraction. It also reduces the prozone. Upon further absorption, however, a decrease in agglutination of Rh positive cells occurs, suggesting that following the removal of the blocking variety of Rh agglutinins the incomplete (albumin) variety of Rh agglutinins is removed next.

In order to study the specificity of the blocking effect of the "supernatant fraction" of serum (Ree) upon the agglutination of Rh-positive cells, the following experiment was set up.

A 10 per cent suspension of homozygous Rh<sub>1</sub> (CDe/Ce) Group O cells was mixed with equal volumes of (1) undiluted, (2) 1 10 diluted, and (3) 1 100 diluted "supernatant fractions" respectively of (A) anti Rh serum (Ree), and (B) 1 normal, adult, human serum. The washing and resuspending procedures were identical with those described in detail under Experiment I.

The blood cells treated in this way were tested for agglutination in the following manner.

To 0.05 cc of each of the sensitized cell suspensions was added in Part I, 0.05 cc of an undiluted anti D (Rh<sub>0</sub>) serum, and in Part II, 0.05 cc of an undiluted anti C (Rh') serum. Both anti Rh sera contained Rh antibodies of the complete (saline) variety. The tubes were allowed to remain thirty minutes at room temperature, then were centrifuged and read macroscopically for agglutination as shown in Table V.

Homozygous Rh<sub>1</sub> (CDe/Ce) cells treated with the "supernatant fraction" of serum (Ree) fail to be agglutinated upon the addition of anti D (Rh) serum. However, the addition of anti-C (Rh'-70%) serum brings about agglutination without showing any trace of inhibition.

TABLE V AGGLUTINATION BY ANTI D (RH) AND ANTI C (RH) SERA OF RH POSITIVE CELLS TREATED WITH THE "SUPERNATANT FRACTION OF SERUM (REE)

SUPERNATANT FRACTIONS	PART I ANTI D (RH) SERUM		PART II ANTI C (RH) SERUM	
	A SERUM (RBC)	B NORMAL SERUM	A SERUM (RBC)	B NORMAL SERUM
1 Undiluted	-	++++	++++	++++
2 1 10	-	++++	++++	++++
3 1 100	+	++++	++++	++++

All dilutions made with undiluted normal adult human serum of Group O

A similar type of experiment carried out with Rh<sub>1</sub> (cDE/c) cells using an anti E (Rh'') serum proved that agglutination of Rh cells by an anti E antibody was not inhibited by the supernatant fraction of serum (Ree). The blocking effect of the supernatant fraction of serum (Ree) therefore corresponds to the specificity of an anti D (Rh) antibody. These results parallel the findings obtained by Hittersley and Fawcett<sup>4</sup> in their study of the prozone phenomena shown by several anti Rh sera.

#### DISCUSSION

The experiments described in this paper were designed to analyze the nature of the prozone phenomenon of the 'supernatant fraction' obtained by dialysis from a certain anti Rh serum (Ree). The question presented itself as to whether this prozone was caused by a surplus of Rh antibodies or was due to the presence of a new and different type of Rh antibody which inhibited rather than produced agglutination. Rh positive cells after being exposed to the 'supernatant fraction' of serum (Ree) in concentrations which gave the prozone effect should have become agglutinated following removal of an excess of Rh antibodies by multiple washings. Rh positive cells treated in this manner, however, did not only fail to become agglutinated when resuspended in saline solution or undiluted normal adult human serum but also could not be clumped any more by the addition of Rh antisera containing Rh antibodies of either the complete (saline) or the incomplete (albumin) varieties. It is difficult to avoid the conclusion that the prozone phenomenon under discussion must be caused by an Rh antibody of blocking character rather than by the presence of a surplus of Rh antibodies of the two known varieties (complete and incomplete).

Furthermore maximum agglutination of Rh positive cells (4 plus agglutination) should have occurred upon serial dilution of the 'supernatant fraction' of serum (Ree) provided the prozone phenomenon was due only to an excess of Rh antibodies and not to the presence of a particular blocking antibody. This fraction, however, never produced agglutination of the 4 plus strength yet 4 plus agglutination occurred following its absorption with Rh positive cells. The new type of Rh antibody herein described must therefore possess a greater avidity for the Rh antigen of the red blood cell than the incomplete (albumin) Rh antibody. This latter antibody in turn is evidenced by the experiments of Coombs and Rice<sup>5</sup> and Levine and Waller<sup>6</sup> has a greater avidity

for the Rh antigen than the complete (saline) Rh antibody. In other words, there is a difference in the speed of combination between the various types of Rh antibodies and the Rh antigen.

In addition to differences in the speed of union between Rh antigen and the different Rh antibodies, there seems also to be a difference in the strength of the Rh antigen-antibody combinations. Multiple washings with saline solution removed the incomplete (albumin) variety of Rh antibody from Rh positive cells in contrast to the new type of Rh antibody which remained attached to the cells, apparently not being influenced by the multiple washing procedure.

#### CONCLUSIONS

1 The prozone phenomenon observed in the "supernatant fraction" obtained by dialysis from an anti-Rh serum (Ree) was found to be due to a new (third) type of Rh antibody rather than to a surplus of Rh antibodies of known varieties.

2 This new type of Rh antibody, when mixed with Rh positive cells prevented their agglutination upon the subsequent addition of Rh antisera containing Rh agglutinins of both the complete (saline) and the incomplete (albumin) variety.

3 In contrast, the "supernatant fractions" obtained by dialysis from sera containing the incomplete (albumin) variety of Rh antibodies, as well as from normal sera, failed to reveal any inhibitory effect on the agglutination of Rh positive cells.

4 The third type of Rh antibody could not be removed from Rh positive cells by multiple washings with saline solution, in contrast to the incomplete (albumin) type of Rh antibody which can be eluted by a series of washings.

5 Absorption of the "supernatant fraction" of serum (Ree) with equal volumes of Rh-positive cells increased rather than decreased the agglutinating properties of this fraction. In contrast, the agglutinin titer of a "supernatant fraction" containing only the incomplete (albumin) type of Rh antibody was reduced materially by this treatment.

6 The third Rh antibody described was found to have anti D (Rh-) specificity.

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## STUDIES ON RH ANTIBODIES

### III ANALYSIS OF A ZONE PHENOMENON IN AN RH ANTISERUM SPLIT BY DIALYSIS INTO FOUR FRACTIONS

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THE observations reported in the two preceding papers<sup>1, 2</sup> dealt with the anti Rh serum (Ree) and its two fractions obtained by dialysis for eighteen hours following the method described previously.<sup>3</sup> However the amount of precipitate which formed during this period varied for different sera. It was influenced by the character of the serum itself such as its lipid content and depended, to a certain extent at least upon the amount of distilled water used the number of changes of distilled water made during each dialysis period and other factors. Furthermore, after removal of the first precipitate which formed after eighteen hours additional precipitation occurred following continued dialysis. Assuming that precipitates formed during various stages of dialysis would also correspond to different globulin fractions of the serum their antibody content was examined. The method used constituted a further division of the original serum into four fractions (three globulin fractions and one supernatant fraction<sup>4</sup>) in contrast to the previous dialysis technique which allowed the splitting of anti Rh sera into two fractions only (the globulin fraction<sup>5</sup> and the 'supernatant fraction<sup>6</sup>'). This more refined method of dialysis of anti Rh sera revealed results which are the subject of this report.

#### TECHNIQUE

All of the dialysis experiments reported in this and in the previous communications were performed using the same type of dialysis membrane which was prepared by cutting a sufficient length of viscose tubing, using  $\frac{7}{32}$  inch casing of a size approximately 43 mm in diameter. A bag was made from the piece of tubing by folding one end upon it self several times and securing it tightly with twine. The tubing was opened by moistening it with distilled water, and was filled with distilled water testing for leakage by placing the contents of the bag under as great a tension as possible. The respective serum specimen to be examined which was stored frozen at  $-30^{\circ}\text{C}$  was thawed at room temperature. Using a small glass funnel 100 cc of this serum were poured into the viscose tubing bag. The air was forced out of the membrane without appreciable loss of the serum and the open end of the bag was twisted tightly in order to put the serum under tension. This end then was secured firmly with twine. After weighting the bottom end of the dialyzing bag with a one hole rubber stopper, the outside was rinsed thoroughly with distilled water. The bag then was suspended from a glass stirring rod into a battery jar holding 12,000 cc of freshly distilled water pre-cooled to  $4^{\circ}\text{C}$  by storage in the refrigerator for at least four hours prior to dialysis. Dialysis was allowed to continue in this first battery jar in the refrigerator at  $4^{\circ}\text{C}$  for six hours. After this period the dialyzing bag was transferred to a second battery jar also containing

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The viscose tubing consists of regenerated cellulose and is made commercially by the Visking Corporation Chicago, Ill. It is obtainable from laboratory supply houses such as the Fisher Scientific Company, Pittsburgh, Pa. or the Will Corporation, Rochester, N. Y.

12,000 cc of freshly distilled water precooled to 4° C, and dialysis was permitted to continue in the refrigerator for twelve hours. The total time period allotted for this first phase of dialysis was eighteen hours.

The bag then was inverted several times, resuspending completely the precipitate which had settled to the bottom, and the exterior of the membrane was rinsed with distilled water. The bottom of the bag was opened by severing the string and the contents were emptied into a large glass beaker.

Approximately half of the dialysate was transferred to a plastic centrifuge tube of 80 cc capacity and centrifuged in an angle centrifuge at 3,000 revolutions per minute for thirty minutes. The supernatant was decanted off and saved. The remaining half of the dialysate was added to the same centrifuge tube containing the sediment obtained in the previous run and spun down, thus collecting all of the precipitate in the one centrifuge tube. The supernatants were combined as "supernatant fraction" and saved for further dialysis. The tube containing the packed sediment was inverted on coarse filter paper and allowed to drain for thirty minutes at room temperature.

The precipitate recovered from the 100 cc of serum was dissolved in 10 cc of 0.9 per cent saline solution which was added in divided amounts. After each portion of saline solution was added, the precipitate was stirred vigorously with a glass stirring rod. Final solution occurred readily at room temperature within fifteen to thirty minutes. The globulin solution was transferred to a double thickness, Pyrex, conical centrifuge tube and spun down in an angle centrifuge for fifteen minutes at 1,500 revolutions per minute. Only a slight amount of insoluble material remained and this was discarded. The clear solution was lyophilized in 1 cc amounts and stored at room temperature. This material is referred to as "globulin fraction No. 1" which represents a ten times concentration as compared with the native serum.

The "supernatant fraction" obtained from this first phase of dialysis was again subjected to eighteen hours of dialysis exactly as described for the first period. The precipitate which formed was collected in the same manner as stated for "globulin fraction No. 1." Inasmuch as it was slightly less in amount than the first precipitate, it was dissolved in only 5 cc of 0.9 per cent saline solution. The resulting solution was labelled "globulin fraction No. 2," constituting a twenty times concentration as compared with the native serum.

The "supernatant fraction" recovered following the second stage of dialysis was dialyzed a third time over a period of eighteen hours as already described in detail. The small amount of precipitate obtained was dissolved in 2 cc of physiologic saline solution. This was labelled "globulin fraction No. 3" and amounts to a fifty times concentration as compared with the native serum.

The final supernatant fluid recovered from the third dialysis was lyophilized in 10 cc amounts and is referred to as the "supernatant fraction." When employed for experimental purposes the dried "globulin fractions" were dissolved in distilled water and the salt free "supernatant fraction" in 0.9 per cent saline solution in order to maintain isotonicity in all fractions.

#### EXPERIMENTAL

The Rh antibody content of the four fractions derived from serum (Ree) was determined by means of quantitative titrations in an experiment which was carried out in two parts. In Part I, all dilutions were made in physiologic saline solution, while in Part II, undiluted, normal, adult, human serum was used as a diluent. The experiment itself was performed as follows.

Decreasing amounts of (A) native serum, (B) "globulin fraction No. 1," (C) "globulin fraction No. 2," (D) "globulin fraction No. 3," (E) "supernatant fraction," volume 0.05 cc each were mixed with 0.05 cc of a 2 per cent suspension of washed Rh (cDE/c) group O cells. The tubes were shaken well, kept for one hour at room temperature and centrifuged for two minutes at 1,500 revolutions per minute. The resulting agglutination is shown in Table I.

TABLE I AGGLUTINATION OF RH POSITIVE GROUP O CELLS BY SERUM (REE) AND ITS FRACTIONS

DILUENT	PART I 0.9% SALINE						PART II UNDILUTED HUMAN SERUM				
	A NATIVE SERUM	B GLOBULIN FRACTION NO 1	C GLOBULIN FRACTION NO 2	D GLOBULIN FRACTION NO 3	E SULFONA TANT FRACTION		A NATIVE SERUM	B GLOBULIN FRACTION NO 1	C GLOBULIN FRACTION NO 2	D GLOBULIN FRACTION NO 3	E SULFONA TANT FRACTION
1 Undiluted	+	+++	-	-	-		++	+++	+++	+++	-
2 1 2	+	+++	-	-	-		+++	+++	+++	+++	-
3 1 4	-	+++	-	-	-		+++	+++	+	+++	-
4 1 8	-	+++	-	-	-		+++	+++	+	+++	-
5 1 16	-	+++	-	-	-		+	+++	+	+++	+
6 1 32	-	+++	-	-	-		+	+++	+	+++	+
7 1 64	-	+	-	-	-		+	+++	+	+++	+
8 1 128	-	+	-	-	-		+	+	-	+	+
9 1 256	-	-	-	-	-		+	+	-	+	+
10 1 512	-	-	-	-	-		+	+	-	+	+
11 1 1024	-	-	-	-	-		+	+	-	+	+
12 1 2048	-	-	-	-	-		+	-	-	-	+
13 0	-	-	-	-	-		-	-	-	-	-

- No agglutination  
 + Faint agglutination  
 + Slight agglutination  
 ++ Marked agglutination  
 +++ Strong agglutination  
 ++++ Very strong agglutination

"Globulin fraction No 1" contains a potent complete (saline) Rh agglutinin. "Globulin fractions No 2 and No 3" as well as the "supernatant fraction" are devoid of any complete (saline) Rh antibodies. It should be kept in mind that the "globulin fractions" compared with the native serum are 10, 20, and 50 times concentrated respectively. When undiluted, human serum is used as a diluent, the native serum reveals the presence of Rh agglutinins, again exhibiting a definite zone phenomenon in the middle of the series of dilutions. "Globulin fractions No 2 and No 3" contain Rh antibodies of the incomplete (albumin) variety, decreasing in strength in a straight line upon dilution, indicating that it is possible to separate the complete and the incomplete Rh antibodies by dialysis. Obviously, therefore, prolonged dialysis of the serum has resulted finally in the precipitation of "globulin fractions" containing the incomplete (albumin) Rh antibodies also.

This experiment might well be compared with the Experiment I described in the first paper of this series.<sup>1</sup> The single eighteen-hour dialysis did not bring about the precipitation of the incomplete (albumin) Rh agglutinins which remained in solution. Nevertheless, even after three eighteen hour dialysis periods, a certain amount of incomplete (albumin) Rh antibodies still remains in the "supernatant fraction" which exhibits a strong prozone phenomenon.

The question arose whether the "supernatant fraction" remaining after dialysis of serum (Rec) for three days would still contain the blocking antibody described in the preceding communication.<sup>2</sup> In order to clarify this point the following experiment was carried out.

#### I Method of Sensitization of Rh Positive Cells —

- 1 0.5 cc of a 10 per cent suspension of three times washed Rh<sub>-</sub> (cDE/c) Group O cells was added to four rows of six tubes each.
- 2 Increasing dilutions (1) undiluted, (2) 1:3, (3) 1:9, (4) 1:27, (5) 1:81, and (6) 1:243 of the following materials were added in a volume of 0.5 cc per tube as follows:
 

Row (A)	Native (untreated) serum (Rec)
Row (B)	"Supernatant fraction" of serum (Rec)
Row (C)	Normal adult serum
Row (D)	"Supernatant fraction" of a normal adult serum
- 3 After shaking the tubes they were kept for thirty minutes in the refrigerator at 4° C.
- 4 The tubes were centrifuged for ten minutes at 4,500 revolutions per minute and the supernatants were aspirated and discarded.
- 5 The sediments, consisting of packed red blood cells, were washed thoroughly three times, using 5 cc of ice cold 0.9 per cent saline solution per tube.
- 6 Following the third washing, all of the saline solution was removed by aspiration.
- 7 0.5 cc of undiluted, normal, adult, human serum was added to each tube and the cells were resuspended completely in the serum.

II Method of Testing the Sensitized Rh Positive Cells for Agglutination With Anti Rh Sera—The experiment was set up in triplicate. To 0.05 cc of the cell suspensions of each of the tubes of rows (A) to (D), pipetted into a new series of small tubes, was added in Part I, 0.05 cc of undiluted anti D (Rh<sub>0</sub>) serum containing Rh agglutinins of the complete (saline) variety, in Part II, 0.05 cc of undiluted anti D (Rh<sub>0</sub>) serum containing Rh agglutinins of the incomplete (albumin) variety, and in Part III, 0.05 cc of undiluted, normal, adult, human serum. After standing for forty five minutes at room temperature the tubes were centrifuged for two minutes at 1,500 revolutions per minute. The resulting agglutinations are seen in Table II.



TABLE II AGGLUTINATION BY ANTI RH SERA OF RH POSITIVE GROUP O CELLS PREVIOUSLY TREATED WITH NATIVE SERUM (REE) AND ITS "SUPERNATANT FRACTION"

ANTI RH SERUM	PART I				PART II				PART III			
	ANTI D (RH) SERUM—COMPLETE VARIETY		ANTI D (RH) SERUM—INCOMPLETE VARIETY		ANTI D (RH) SERUM—INCOMPLETE VARIETY		UNDILUTED NORMAL ADULT SERUM (CONTROL)		UNDILUTED NORMAL ADULT SERUM (CONTROL)		UNDILUTED NORMAL ADULT SERUM (CONTROL)	
SENSITIZING SERUM	SERUM SUPERNATANT (REE)		SERUM SUPERNATANT (PEE)		SERUM SUPERNATANT (PEE)		SERUM SUPERNATANT (PEE)		SERUM SUPERNATANT (PEE)		SERUM SUPERNATANT (PEE)	
	A	B	C	D	A	B	C	D	A	B	C	D
1 Undiluted	+	-	+++	+++	+++	+++	+++	+++	+	-	-	-
2 1	+	-	+++	+++	+++	+++	+++	+++	+	-	-	-
3 1 9	-	-	+++	+++	+++	+++	+++	+++	-	-	-	-
4 1 27	-	+	+++	+++	+++	+++	+++	+++	-	-	-	-
5 1 81	+	++	+++	+++	+++	+++	+++	+++	-	-	-	-
6 1 -43	++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-

All dilutions made with undiluted normal adult human serum of Group O

As can be seen from Experiment II, blocking antibodies are present in the "supernatant fraction" of serum (Ree). The inhibitory effect of the "supernatant fraction" manifests itself against both the complete (IB) and the incomplete (IIB) varieties of anti-D ( $Rh_0$ ) antibodies, though stronger against the complete (saline) Rh agglutinins. The native serum (Ree), too, inhibits the complete (saline) anti-Rh agglutinins (IA). Strangely enough, however, it fails to inhibit the agglutination of Rh-positive cells by the incomplete (albumin) variety of anti-Rh agglutinins (IIA). The failure of Rh positive cells previously sensitized by the native serum (Ree) to be agglutinated when taken up in undiluted, normal, adult, human serum (IIIA)\* confirms the observations made in the preceding paper<sup>2</sup> that the incomplete (albumin) variety of Rh antibody can be removed from sensitized cells, to a great extent at least, by multiple washings.

An attempt now was made to demonstrate in the native serum proper the presence of blocking antibodies against the incomplete (albumin) Rh antibody by subjecting Rh-positive cells sensitized by the native serum to a more thorough washing procedure than that used in the preceding experiment. To this end the following experiment was carried out.

#### I Method of Sensitization of Rh Positive Cells —

- 1 0.1 cc of a 10 per cent suspension of three times washed heterozygous Rh, (cDE/c) Group O cells was added to eight rows consisting of ten tubes each.
- 2 Increasing dilutions (1) undiluted, (2) 1/3, (3) 1/9, (4) 1/27, (5) 1/81, (6) 1/243, (7) 1/729, (8) 1/2,187, (9) 1/6,561 and (10) 1/19,683 of the following materials were added in a volume of 0.1 cc per tube as follows:
 

Row (A)	Native (untreated) serum (Ree)
Row (B)	"Supernatant fraction" of serum (Ree)
Row (C)	Normal, adult serum
Row (D)	"Supernatant fraction" of a normal, adult serum

The experiment was set up in duplicate.

- 3 The tubes were shaken and kept for thirty minutes at 4° C.
- 4 After centrifuging the tubes for ten minutes at 3,000 revolutions per minute the supernatants were aspirated and discarded.
- 5 The sediments, consisting of packed red blood cells, were washed thoroughly four times using 2 cc of ice cold 0.9 per cent saline solution per tube.
- 6 After the fourth washing all of the saline solution was removed completely by aspiration.
- 7 0.1 cc of undiluted, normal, adult, human serum was added to each tube and the cells were resuspended in the serum.

II Method of Testing the Sensitized Rh Positive Cells for Agglutination with Anti Rh Sera — In Part I, 0.1 cc of 1/3 diluted anti D ( $Rh_0$ ) serum containing Rh agglutinins of the incomplete (albumin) variety was added to each of the tubes, and in Part II, 0.1 cc of undiluted, normal, adult, human serum was added to each of the tubes. The tubes were allowed to stand at room temperature for one hour and were centrifuged for two minutes at 3,000 revolutions per minute. The agglutination as read macroscopically is recorded in Table III.

\*Weak agglutination obtained in the first two tubes in Row (A) of Part I can be explained by the presence of traces of Rh antibodies not yet completely removed by washing as indicated by an identical reaction in the first two tubes of Row (A) of Part III in which undiluted normal adult, human serum was used as a control in place of anti Rh serum.

TABLE III AGGLUTINATION BY AN ANTI RH SERUM OF THE INCOMPLETE VARIETY OF RH POSITIVE GROUP O CELLS PREVIOUSLY TREATED WITH NATIVE SERUM (REE) AND ITS ' SUPERNATANT FRACTION

ANTI RH SERUM	PART I ANTI D (RH) SERUM— INCOMPLETE VARIETY				PART II UNDILUTED NORMAL ADULT SERUM (CONTROL)			
	A		C		A		C	
	SERUM	SUPERNATANT (REE)	SERUM	SUPERNATANT NORMAL	SERUM	SUPERNATANT (REE)	SERUM	SUPERNATANT NORMAL
1 Undiluted	+	-	+++	+++	+	-	-	-
1 3	±	-	+++	+++	±	-	-	-
3 1 9	-	-	+++	+++	-	-	-	-
4 1 9	-	±	+++	+++	-	-	-	-
5 1 81	-	+	+++	+++	-	-	-	-
6 1 243	±	++	+++	+++	-	-	-	-
7 1 729	+	+++	+++	+++	-	-	-	-
8 1 2187	+++	+++	+++	+++	-	-	-	-
9 1 6561	+++	+++	+++	+++	-	-	-	-
10 1 19683	+++	+++	+++	+++	-	-	-	-

All dilutions made with undiluted normal adult human serum of Group O

Experiment III shows that blocking antibodies against the incomplete (albumin) Rh antibody can be demonstrated even in the native serum (Ree). Their demonstration is made possible only by thoroughly washing the sensitized Rh positive cells a procedure which apparently is necessary to remove Rh antibodies other than the blocking variety which remains attached to the cells in spite of the washing.

Coombs Mourant, and Race<sup>4,6</sup> have described an important technique which permits the detection of sensitization of Rh positive cells by Rh antibodies. Rh positive cells sensitized by Rh antibodies without showing visible agglutination are washed several times and then agglutinated by the addition of an antihuman serum rabbit serum. It was of interest to find out which position the Coombs test would assume in the detection of Rh antibodies as found in the various fractions of the Rh antiserum (Ree). The following experiment was carried out to elucidate this problem.

#### I. Method of Sensitization of Rh Positive Cells—

- 0.1 cc of a 10 per cent suspension of washed heterozygous Rh (CDe/ce) cells belonging to blood Group O was added to each of twelve tubes in six rows.
- Increasing dilutions (1) undiluted (2) 1 3 (3) 1 9 (4) 1 27 (5) 1 81, (6) 1 243 (7) 1 729, (8) 1 2187, (9) 1 6561 (10) 1 19683, (11) 1 59049, and (12) 1 177147 of the following materials were added in a volume of 0.1 cc per tube as follows:

- Row (A) Native (untreated) serum (Ree)
- Row (B) ' Globulin fraction No 3 ' of serum (Ree)
- Row (C) ' Supernatant fraction of serum (Ree)

These titrations were performed in duplicate.

- The tubes were shaken and kept for thirty minutes in the refrigerator at 4°C.
- Following centrifugation for fifteen minutes at 3000 revolutions per minute the supernatants were aspirated from the tubes and discarded.
- The packed red blood cells in each tube were washed three times using 2 cc of ice cold 0.9 per cent saline solution per tube.

- 6 After the third washing all of the saline solution was removed as completely as possible by aspiration
- 7 In Part I, 0.1 cc of 0.9 per cent saline solution was added to each of the tubes, while in Part II, 0.1 cc of undiluted, normal, adult, human serum was added to each of the tubes and the cells were resuspended in the respective diluents

**II Method of Testing the Sensitized Rh Positive Cells for Agglutination With Antihuman Serum Rabbit Serum**—In Part I, 0.1 cc of 1:30 diluted antihuman serum rabbit serum was added to each of the tubes, and in Part II, an additional 0.1 cc of the same undiluted, normal, adult, human serum used for resuspending the cells was added to each of the tubes (control). The tubes were allowed to stand at room temperature for one hour and then were centrifuged for two minutes at 1,500 revolutions per minute. The resulting agglutination are seen in Table IV.

TABLE IV. COOMBS TEST

AGGLUTINATION BY ANTIHUMAN SERUM RABBIT SERUM OF RH POSITIVE CELLS SENSITIZED WITH NATIVE SERUM (REE) AND TWO OF ITS FRACTIONS RESPECTIVELY

ANTISERA	PART I ANTHUMAN SERUM RABBIT SERUM			PART II UNDILUTED, NORMAL, ADULT SERUM (CONTROL)		
	A NATIVE SERUM	B GLOBULIN FRACTION NO 3	C SUPERNATANT FRACTION	A NATIVE SERUM	B GLOBULIN FRACTION NO 3	C SUPERNATANT FRACTION
1 Undiluted	++++	++	++++	-	-	-
2 1:3	++++	++++	++++	-	-	-
3 1:9	++++	++++	++++	-	-	-
4 1:27	++++	++++	++++	-	-	-
5 1:81	++++	++++	+++	-	-	-
6 1:243	++++	++++	++	-	-	-
7 1:729	++++	++++	+	-	-	-
8 1:2187	++++	++++	-	-	-	-
9 1:6561	+++	++++	-	-	-	-
10 1:19,683	+++	++++	-	-	-	-
11 1:59,049	++	++	-	-	-	-
12 1:177,147	+	++	-	-	-	-

The exceeding sensitivity of the Coombs test is evident from this experiment. It allows the detection of the sensitization of Rh-positive cells by the native serum (Ree) up to the final dilution used (up to 1:177,000). The "supernatant fraction" of the serum (Ree) containing blocking Rh antibodies gives a positive Coombs test up to a dilution of 1:729, roughly corresponding to its content of blocking Rh antibody (see Experiment III). The strength of the Coombs test obtained with "globulin fraction No. 3" is surprising. This "globulin fraction" contained Rh antibodies of the incomplete (albumin) variety demonstrable up to dilutions not higher than 1:256 (see Experiment I) and yet the Coombs test is still definitely positive with the highest dilution used in this experiment (1:177,000). This finding is even more surprising if one considers the results of Part II of this experiment. Rh-positive test cells treated in an exactly identical way as in Part I were resuspended in undiluted adult serum without showing any trace of agglutination, pointing to the fact that the incomplete (albumin) Rh antibodies originally present were washed away or at least rendered ineffective by the washing procedure. It should also be mentioned that in experiments not reported in this paper, "globulin fraction No. 3" was found to be devoid completely of Rh blocking antibodies.

## DISCUSSION

Dialysis has been used during the last three years in this laboratory as a means of splitting anti Rh sera into two fractions namely the precipitate referred to as the globulin fraction and the supernatant fraction which was obtained following removal of the precipitate by centrifugation. As previously reported<sup>3</sup> the precipitate frequently contained the complete (saline) Rh antibody while the incomplete (albumin) Rh antibody was found in the supernatant. In this manner certain anti Rh sera which were useless as test sera could be made into potent reagents for Rh determinations using the globulin fraction. This fraction can be concentrated easily depending upon the volume of saline solution in which it is dissolved. The method also proved that Rh antibodies of different varieties might be separated from each other because they apparently are associated with different protein fractions of serum.

In this paper a more quantitative method was used. Dialysis was continued over a period of three days during which time precipitates formed after each eighteen hour period were collected separately and examined for their Rh antibody content. Prolonged dialysis finally led to the precipitation of a good portion of the incomplete (albumin) variety of Rh antibody. However the remaining supernatant fluid still contained in Rh antibody with typical blocking qualities. It seems doubtful whether further dialysis would have resulted also in precipitation of this type of Rh antibody. It should be interesting to find out how the method of fractionated alcohol precipitation, as carried out by Cohn and associates compares with the relatively simple procedure of dialysis used in the investigations reported. It seems unlikely that dialysis in the cold denatures proteins at all which might give this method a certain advantage over others.

Differences in avidity among the various types of Rh antibodies manifest themselves upon washing sensitized Rh positive cells. The incomplete (albumin) variety of Rh antibody can be rather easily washed off with three or four thorough washings as proved by the fact that Rh positive cells previously sensitized with this type of Rh antibody and then washed failed to be agglutinated when suspended in undiluted adult human serum or albumin solution respectively. It was not possible, however, to remove the blocking type of Rh antibody present in the 'supernatant fraction' of serum (Ree) even by a series of thorough washings. As a matter of fact, the blocking effect of the native serum (Ree) became apparent only following washing procedures. As one might speculate the blocking type of Rh antibody tiles over and readily combines with the Rh antigens which become available following the removal of the incomplete (albumin) Rh antibodies.

The name blocking antibody used in this paper denotes an Rh antibody which not only fails to agglutinate but even interferes with the agglutination of Rh positive cells when they are suspended in an albumin containing medium. It should be recalled that Wiener previously called the incomplete (albumin) variety of Rh antibody the 'blocking' antibody because it prevented agglutination of Rh positive cells by the complete (saline) variety of Rh antibody in

physiologic saline solution. However, this blocking effect of the incomplete (albumin) Rh antibody was evident only when physiologic saline solution was used as a diluent, but not in undiluted serum or in albumin solution. The blocking antibody described in this paper inhibits agglutination of Rh positive cells even in an albumin-containing medium and it truly blocks the incomplete (albumin) variety of Rh antibody as well as the complete (saline) variety.

Therefore, the existence of three varieties of Rh antibodies can be assumed: (1) the complete or saline variety, (2) the incomplete or albumin variety, and (3) the blocking variety of Rh antibody. On the basis of their studies, Hill and Haberman<sup>8,9</sup> already felt that there were three types of Rh antibodies, namely (1) the classical agglutinin, (2) the "blocking" antibody (as determined by Wiener<sup>1</sup>), and (3) the "erythroagglutinoid" (as determined by the developing test<sup>8,9</sup> or Coombs test<sup>10</sup>). The experiments reported in this paper, moreover, point to the likelihood that there is a fourth type of Rh antibody for the following reasons. Experiment IV of this paper reveals the rich content of Coombs antibody in 'globulin fraction No. 3'. This 'globulin fraction No. 3' also contained an incomplete (albumin) Rh antibody with a titer of 256. However, following multiple washings of Rh-positive cells sensitized with this 'globulin fraction,' no agglutination was demonstrable when the washed, sensitized cells were suspended in undiluted, normal, adult serum. Apparently washing had removed the incomplete (albumin) Rh antibody. Nevertheless, the Coombs test carried out with washed Rh-positive cells sensitized with the 'globulin fraction No. 3' showed the presence of an Rh antibody with a titer of at least 177,000. This observation strongly suggests, therefore, that we are dealing with a type of Rh antibody which neither agglutinates Rh positive cells nor exhibits a blocking effect. Still the possibility cannot be overlooked entirely that traces of incomplete (albumin) Rh antibodies, by themselves insufficient in strength to agglutinate the cells but sufficient to give a positive Coombs test, were still present and attached to the Rh-positive cells under investigation. However, the latter explanation is unlikely considering the difference in titer between the incomplete (albumin) Rh antibody on the one hand and the Coombs antibody on the other.

There seems to be a reciprocal relationship between the capacity of the various types of Rh antibody to agglutinate Rh-positive cells and their ability to be removed by washing, that is to say, Rh antibodies which produce visible agglutination of Rh-positive cells can be removed more easily by washing procedures than Rh antibodies that sensitize Rh-positive cells without producing visible agglutination. Considerable differences in the speed and strength of the union between the various Rh antibodies and Rh antigen must exist.

The so called unitarian conception of antibody functions is adhered to by the majority of immunologists at the present time. The well known various manifestations of antigen-antibody reactions such as lysis, agglutination, precipitation, and so on, are explained by this concept on the basis of physico-chemical differences of the antigen rather than of the antibody, depending mainly upon the size of the antigen and the medium in which the antigen-antibody reaction occurs. Therefore, the same antibody that might produce

precipitation in one instance could cause agglutination or lysis of cells under different experimental conditions. The investigations reported in this series of communications are dealing with antibody functions of identical specificity directed against one antigen only, namely the D (or Rh<sub>0</sub>) antigen present in the same physicochemical state in red blood cells. Yet, antibodies directed against this antigen have been found to be associated with separate protein fractions of the antiserum and can be distinguished from each other by their avidity for this Rh antigen and by their reaction with it under various experimental conditions. It seems difficult to see how the findings reported can conform to the present unitarian concept of antigen antibody reactions which appears to need some revision or amplification.

Two theories present themselves in connection with the differences in the antibody functions reported. The first theory would postulate the existence of three or four varieties of independent Rh antibodies which are sharply separated from each other both by the nature of their functions as well as by their association with different protein fractions of the serum. In contrast the second theory might refuse to accept the existence of sharply separated antibody functions and rather hold to the belief in a gradual change of one antibody function into another, assuming the existence of an Rh antibody spectrum of changing activity.

#### CONCLUSIONS

1 Prolonged dialysis of an anti Rh serum (Ree) against measured amounts of distilled water for three eighteen hour periods resulted in the precipitation of globulins which were collected individually at the end of each eighteen hour period. Three fractions obtained in this way were referred to as globulin fractions No 1, No 2 and No 3 respectively. The clear supernatant fluid remaining at the end of the third period of dialysis was called the 'supernatant fraction'.

2 Globulin fraction No 1 revealed the presence of a complete (saline) anti Rh agglutinin which was not found in any other fraction.

3 Globulin fractions No 2 and No 3 contained an Rh antibody of the incomplete (albumin) variety.

4 The supernatant fraction still containing Rh agglutinins of the incomplete (albumin) variety, exhibited a strong prozone phenomenon.

5 The prozone phenomenon of the supernatant fraction is attributed to the presence of a peculiar Rh antibody which inhibits agglutination of Rh positive cells by both the complete (saline) and the incomplete (albumin) variety of Rh antibodies. It acts therefore, as a true blocking antibody (third variety of Rh antibody).

6 The presence of this Rh blocking antibody also could be demonstrated in the untreated native anti Rh serum (Ree) by subjecting Rh positive cells sensitized with this serum to thorough washing procedures which apparently resulted in the removal of the incomplete (albumin) variety of Rh antibody.

7 Rh positive cells sensitized with globulin fraction No 3 failed to show macroscopically visible agglutination after being washed thoroughly and

suspended in undiluted, normal, adult, human serum. This fraction contained only incomplete (albumin) Rh antibodies of low titer and was devoid of Rh blocking antibodies. Nevertheless, cells sensitized with it were strongly agglutinated following the addition of antihuman serum rabbit serum (Coombs test), suggesting the existence of a fourth variety of Rh antibody.

8. The occurrence in one anti-Rh serum of certainly three and possibly four varieties of Rh antibodies of the same specificity, anti-D ( $Rh_o$ ), but of different functions is described. They produce at the extremes of the spectrum of their activity either agglutination or blocking of Rh-positive cells with one variety standing in between which causes neither phenomenon, being demonstrable only by means of the Coombs test. The possible significance of these findings in relation to the validity of the present unitarian concept of antibody functions is discussed.

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# METABOLISM TOXICITY AND MANNER OF ACTION OF GOLD COMPOUNDS IN THE TREATMENT OF ARTHRITIS

## VIII THE EFFECT OF BAL AND OTHER THIOL COMPOUNDS IN PREVENTING THE INHIBITION OF OXYGEN CONSUMPTION OF RAT TISSUES

PRODUCED BY GOLD SALTS

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BOTH organic and inorganic gold compounds have been used for many years in the treatment of rheumatoid arthritis. The value of this form of treatment is subject to argument and final appraisal on the basis of therapeutic trial apparently is difficult or impossible. The use of gold compounds in rheumatoid arthritis remains on an empiric basis, and even with carefully controlled administration toxic reactions have been a limiting factor in their use. In order to place this form of treatment on a more rational basis, information regarding the manner by which gold salts produce their effects in the body must be available.

An intensive study of the metabolism and toxicity of the various gold compounds used has been made in this laboratory.<sup>1</sup> It was found that when gold compounds are injected intramuscularly into white rats the gold is deposited in various tissues of the body, primarily in the liver and kidney.<sup>2</sup> The amount and site of deposition seem to depend on the physical properties of the compound studied. Also, the severity of the histopathology in gold-treated animals is roughly proportional to the quantity of gold laid down in the tissue.

We became interested in whether the respiration of these tissues as measured by oxygen consumption, is influenced by the presence of gold and whether a correlation exists between the concentration of gold in the tissue and its rate of respiration. If such a relationship between deposition of gold and activity of the enzyme systems in these tissues exists, it may provide an insight into the mechanism of gold toxicity. Using the Warburg technique it was found that the oxygen consumption of rat kidney and liver slices was inhibited *in vitro* by the inorganic compounds gold chloride and gold sodium thiosulfate. The organic compounds sodium succinimido aurate, gold sodium thiomalate and gold thioglucose did not cause inhibition.<sup>3</sup>

When 2,3-dimercaptopropanol (BAL) developed during the war as an effective antidote against acute arsenical poisoning became available, it was shown to be effective also against other heavy metals: cadmium, mercury, zinc and copper.<sup>4</sup> Furthermore, recent reports indicate the apparent clinical value of BAL in treating gold toxicity in human beings and suggest that its administration results in an augmented urinary excretion of gold under these conditions.<sup>5, 6</sup>

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It is now generally accepted that the toxicity of heavy metals, such as arsenic, is due to the inactivation of the sulphydryl groups in enzyme proteins of the tissues. The action of the thiols is attributed to their effective competition for the heavy metal with the sulphydryl groups in various body proteins. Arsenic and gold are closely related chemically and it is reasonable to assume that their biochemical reactions are similar. It is suggested that the toxicity of gold is due to its combination with sulphydryl groups in enzyme proteins, and the sulphydryl (thiol) groups in BAL compete effectively for the gold combined in the tissue protein, thus restoring the normal enzyme system.

This study is concerned with the effect of BAL and other thiol compounds on the respiration of tissues *in vitro*, and their possible effect in preventing inhibition of oxygen consumption produced by gold compounds. Obviously, only those gold compounds observed to produce such inhibition in previous studies could be investigated.

#### METHODS

Measurements of oxygen consumption were made with the usual type of Warburg constant volume manometer. The carbon dioxide produced was absorbed by 0.3 cc of 20 per cent potassium hydroxide solution contained in the central well on pleated filter paper. After introduction of the tissue slice, the manometer flasks were immersed in a water bath at 38° C, flushed with pure warm oxygen (38° C) for ten minutes, and shaken at a rate of 110 oscillations per minute. The suspending medium was a phosphate buffered physiologic salt solution (pH 7.4) containing 0.2 per cent glucose as described by Krebs<sup>18</sup>. The total volume of fluid in each flask was 2.5 cubic centimeters.

The tissues were taken from healthy male and female white rats which had been fed a standard stock diet. The animals were killed by a blow on the head and the kidneys and/or liver were quickly removed, washed free of blood, and placed in the buffer solution. Slices of approximately equal size and thickness were made with a razor blade held against the lower side of a glass microscope slide. The sections then were placed in the main chamber of the manometer flasks. At the conclusion of the experiment the slices were removed and dried for twenty four to forty eight hours at 75° C and oxygen consumption was calculated per milligram of dry weight of tissue.

The solutions to be tested were placed in the side arms at 12.5 times the final concentration and tipped into the main chamber containing the tissues after a control period of forty five minutes. In all cases the final gold concentration was  $M/500$  with respect to gold, since this had been shown to be the lowest concentration which would produce maximal inhibition of oxygen consumption.<sup>6</sup> The thiol compounds were added in concentrations to provide either one thiol or three thiol groups for one atom of gold. This was done since it is possible for gold to react either as a univalent or trivalent ion. The pH of each added solution was adjusted so that the final pH of the solution in the flasks after addition was 7.4.

Each set of experiments included a control tissue slice, one to which gold was added, one with an added thiol compound, and one with both a gold and a thiol compound added. Readings of the manometers were made at fifteen and thirty minute intervals, and were continued from one hour and forty five minutes to two hours and forty five minutes after addition of solutions being tested.

#### OBSERVATIONS

The compounds studied are listed in Table I. The inorganic gold salts, gold chloride and gold sodium thiosulfate, were previously shown to have a marked inhibitory effect on the oxygen consumption of rat tissues.<sup>6</sup> Cystine and methionine were included since it was felt that they might be broken down to give compounds with a thiol group.

TABLE I COMPOUNDS STUDIED

GOLD COMPOUNDS	
$\text{AuCl}$ $\text{HCl}$ $3\text{H}_2\text{O}$ Gold chloride (50 per cent gold)	$\text{Na}_2\text{Au}(\text{S O}_3)_2$ Gold sodium thiosulfate (37.4 per cent gold)
THIOL COMPOUNDS	
$ \begin{array}{c} \text{COOH} \\   \\ \text{H}-\text{C}-\text{SH} \\   \\ \text{H}-\text{C}-\text{H} \\   \\ \text{COOH} \end{array} $ Phiomalic acid	$ \begin{array}{c} \text{H} \\   \\ \text{H}-\text{C}-\text{SH} \\   \\ \text{H}-\text{C}-\text{SH} \\   \\ \text{H}-\text{C}-\text{OH} \\   \\ \text{H} \end{array} $ 2,3-Dimercaptopropanol (BAL)
$ \begin{array}{c} \text{H} \\   \\ \text{H}-\text{C}-\text{SH} \\   \\ \text{H}-\text{C}-\text{NH} \\   \\ \text{COOH} \end{array} $ Cysteine	$ \begin{array}{c} \text{NaSCH} \\   \\ \text{HCOH} \\   \\ \text{HOCH} \\   \\ \text{HCOH} \\   \\ \text{HC} \\   \\ \text{CH OH} \end{array} $ Sodium thioglucose†
POTENTIAL THIOL COMPOUNDS	
$ \begin{array}{c} \text{H} \qquad \qquad \text{H} \\   \qquad \qquad   \\ \text{H}-\text{C}-\text{S}-\text{S}-\text{C}-\text{H} \\   \qquad \qquad   \\ \text{H}-\text{C}-\text{NH}_2 \qquad \text{H}-\text{C}-\text{NH} \\   \qquad \qquad   \\ \text{COOH} \qquad \text{COOH} \end{array} $ 1 Cysteine	$ \begin{array}{c} \text{H} \\   \\ \text{H}-\text{C}-\text{H} \\   \\ \text{S} \\   \\ \text{H}-\text{C}-\text{H} \\   \\ \text{H}-\text{C}-\text{H} \\   \\ \text{H}-\text{C}-\text{NH} \\   \\ \text{COOH} \end{array} $ Methionine

The BAL was obtained from Edgewood Arsenal Md through courtesy of the United States Army

†Sodium thioglucose was furnished by the Schering Corporation Bloomfield N J

Only the results with kidney slices are included in this report. Essentially the same results were obtained with liver slices however since the oxygen consumption of normal liver tissue is smaller than that of kidney tissue the absolute differences noted with the compounds studied are much smaller than those found in the kidney.

Oxygen consumption of rat kidney slices as influenced in vitro by the gold compounds alone, by thiol and dithiol compounds alone and by a combination of each, is shown in Fig 1 for gold sodium thiosulfate and Fig 2 for gold

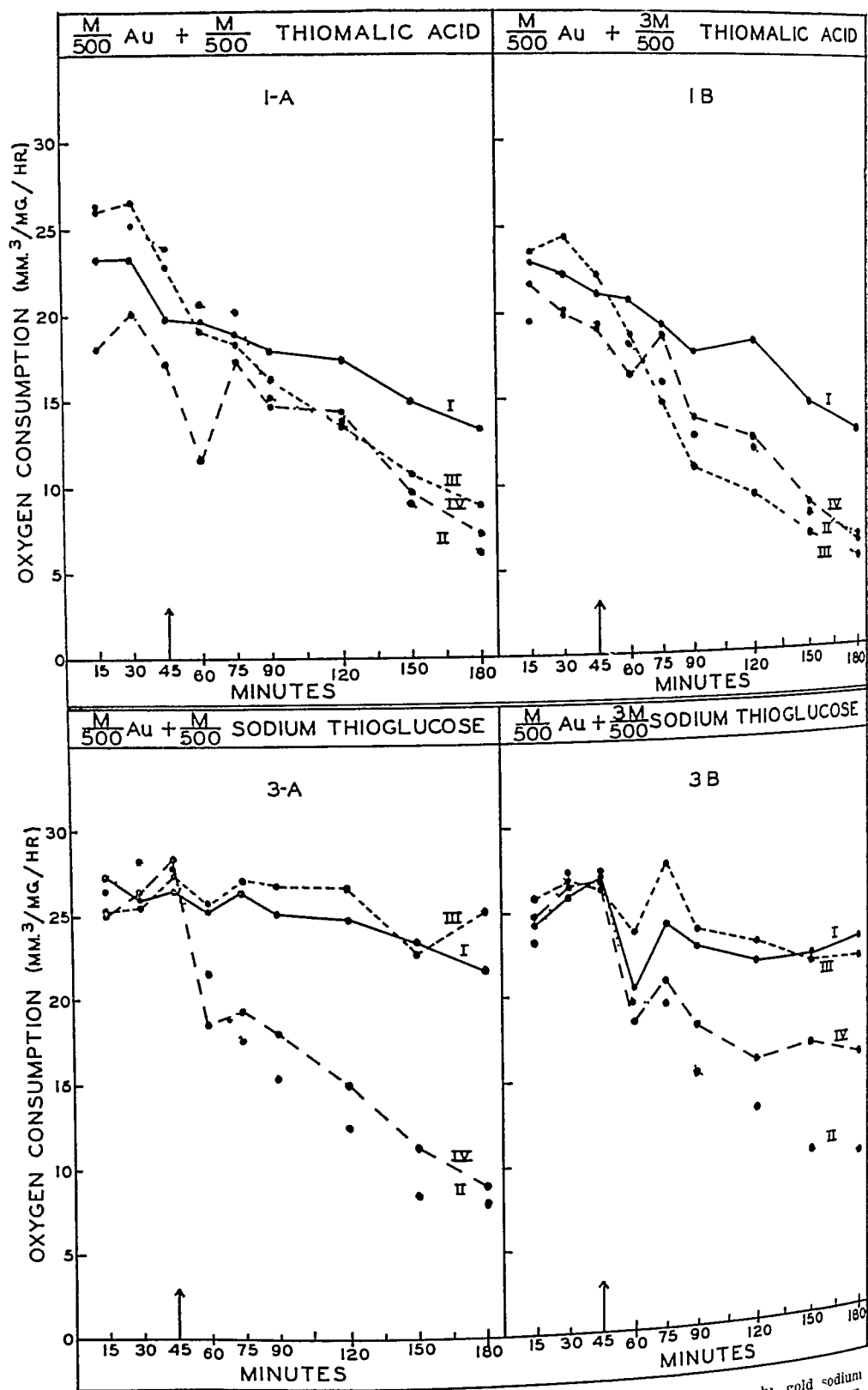


Fig 1—Oxygen consumption of rat kidney slices as influenced in vitro by gold sodium thiosulfate alone by thiol and di-thiol compounds alone and by a combination of each I — No compounds added II — Gold sodium thiosulfate added III --- Thiol compound added IV - - Gold and thiol compounds added The vertical arrow indicates time of addition of compound tested

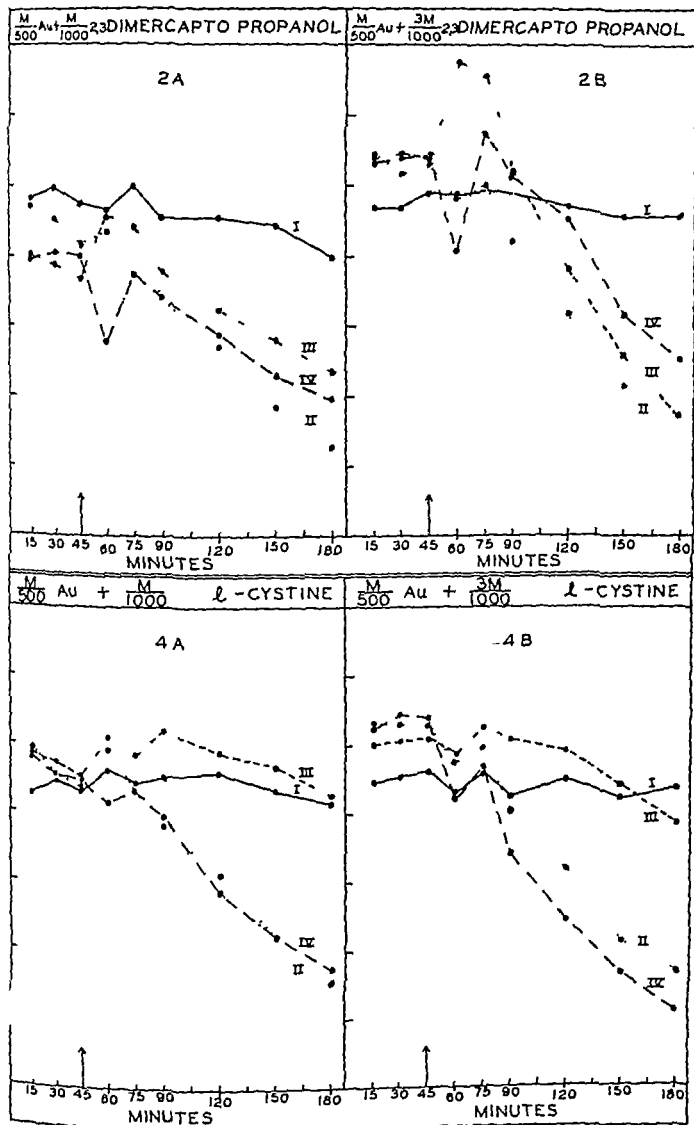


Fig 1—Continued. See legend on opposite page

chloride Oxygen consumption is plotted on the vertical axis against time in minutes on the horizontal axis. The arrow at forty-five minutes indicates the time of additions to the flask. Each curve represents the average of six to eight determinations. The control curve is shown by the solid line, the gold by a dotted line, thiol compound by a broken line, and both compounds by a combination dot-dash line. On the left, in each pair of experiments, the chart shows the results obtained when one thiol group was provided per atom of gold, on the right, the curves represent results with three thiol groups per atom of gold.

*Gold Sodium Thiosulfate (Fig. 1)*—The first pair of curves, 1-A and 1-B, shows the effect of thiomalic acid and gold sodium thiosulfate on oxygen consumption of rat kidney slices. The control curves (I) in both sets of experiments show a gradual decline in respiration over the three-hour period. In the M/500 concentration (1-A) thiomalic acid (III) produces an inhibition of respiration somewhat less than that produced by M/500 gold (II). The addition of both compounds simultaneously to normal tissue results in a respiration (IV) slightly higher than when gold alone is added (II) but lower than with thiomalic acid alone (III). In the 3M/500 concentration (1-B) thiomalic acid itself (III) produces an inhibition of respiration greater than that caused by gold alone (II). However, the simultaneous addition of both compounds results in a curve (IV) which follows very closely that of gold alone (II), indicating that in this instance the inhibition of respiration is not the additive effect of the inhibition produced by each compound alone.

The effect of BAL (2,3 dimercaptopropanol) is shown in 2-A and 2-B. In both sets of experiments the control curves (I) show little decrease in respiration over the whole period. In the M/1,000 concentration (2-A), where one thiol group is provided per atom of gold, the BAL alone (III) produces less inhibition of respiration than does gold alone (II). The curve for the addition of both compounds (IV) shows less inhibition than when gold alone is added (II), but greater inhibition than that caused by the thiol compound alone (III). In the 3M/1,000 (2-B) BAL itself (III) produces an inhibition comparable to that of gold alone (II), however, the rate at which respiration decreases is slower with BAL than with gold. Tissue respiration after the addition of both gold and BAL (IV) is inhibited as compared with the control (I), but continues at a somewhat higher level than with either compound alone. Here again, the inhibitory effect of both compounds is not the sum of the effect of each alone, and it is possible that there is a greater prevention of respiratory inhibition caused by gold which is masked by the individual inhibitory action of either compound.

The influence of sodium thioglucose and gold sodium thiosulfate is shown by the curves in 3-A and 3-B. In concentrations of M/500, equimolar with respect to gold, the thiol compound (III) has no effect when compared with control tissue respiration (I). Its addition together with gold results in a respiration curve (IV) which shows definite inhibition though it is less than that produced by the addition of the gold salt (II). In the 3M/500 concentration, sodium thioglucose produces no inhibition of oxygen consumption (III).

when compared with the control respiration rate (I). However, the addition of both thiol and gold compounds results in a respiration curve (IV) which is significantly higher than that obtained when only gold is added (II).

The last pair of curves, *1 A* and *1 B*, Fig 1 presents results obtained with l-cystine, a potential di thiol compound. In both sets of experiments, cystine itself produces respiration curves (III) which are similar to the control curves (I). In the M/1,000 concentration equimolar with respect to gold, cystine added simultaneously with gold (IV) produces an inhibition of respiration comparable with that caused by the gold salt alone (II). In the 3M/1 000 concentration, the addition of cystine with gold (IV) results in an even lower respiration rate than that produced by gold alone (II). Despite the fact that the pH of all solutions added was adjusted so that the pH of the reaction media was 7.4 after additions to the main chamber, we found that the gold solution plus the 3M/1,000 cystine resulted in a pH of over 8.0 at the conclusion of the experiment. This fact may account in these experiments for the observed respiratory inhibition which is greater than that caused by the gold salt alone.

Methionine, not shown in the figure gave essentially the same results as l-cystine.

*Gold Chloride (Fig 2)*—The *in vitro* effect of gold chloride and the various thiol compounds studied on the respiration of rat kidney slices is shown in Fig 2. The first set of experiments *1 A* and *1 B* shows the influence of thiomalic acid. Alone in M/500 concentration, this thiol compound has a depressant effect on respiration as shown by curve III; however when it is added with the gold salt the respiration rate (IV) is somewhat higher than that produced by the gold alone (II) though lower than with thiomalic acid alone (III). In the 3M/500 concentration (*1 B*), thiomalic acid and the gold salt added simultaneously produce a respiration curve (IV) which shows definitely less inhibition of oxygen consumption than when either compound is added alone. There is however, definite inhibition when this respiration rate is compared with the control (I).

Essentially the same results were obtained with BAL and gold chloride *2 A* and *2 B*, Fig 2 as with thiomalic acid and gold chloride. In the 3M/1 000 concentration (*2 B*), BAL shows a greater inhibition of respiration alone (III) than in the M/1,000 concentration (III *2 A*) but its ability to prevent inhibition is much more marked (IV, *2 B*) at this concentration than at M/1 000 (III *2 A*).

Both sodium thioglucose and l-cystine behave similarly, *3 A* and *3 B*, and *4 A* and *4 B*, respectively. Neither compound produces inhibition of itself (III). Each, when added with the gold chloride in concentrations to provide one thiol group per atom of gold, shows some prevention of inhibition (IV, *3 A* and *4 A*). In the larger concentration, three thiol groups per atom of gold, each compound shows a greater prevention of inhibition (IV) than when only one thiol group is provided per gold atom.

Methionine and cysteine were also studied. Neither produced inhibition of oxygen consumption by itself nor was there any appreciable prevention of the inhibition produced by gold chloride.

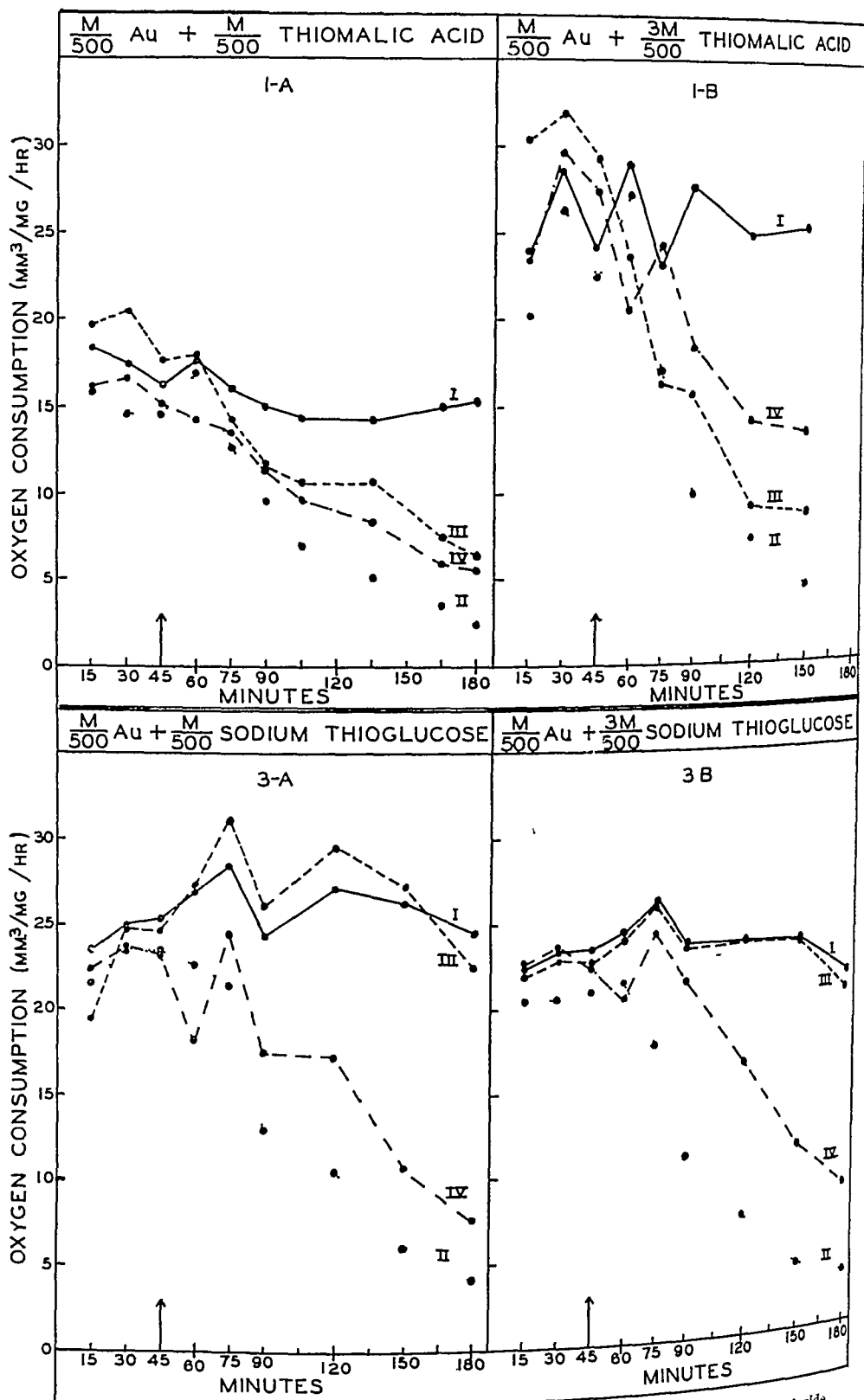


Fig 2—Oxygen consumption of rat kidney slices as influenced in vitro by gold chloride alone by thiol and di-thiol compounds alone and by a combination of each I — No compounds added II — Gold chloride added III — Thiol compounds added IV — Gold and thiol compounds added The vertical arrow indicates time of addition of compound tested



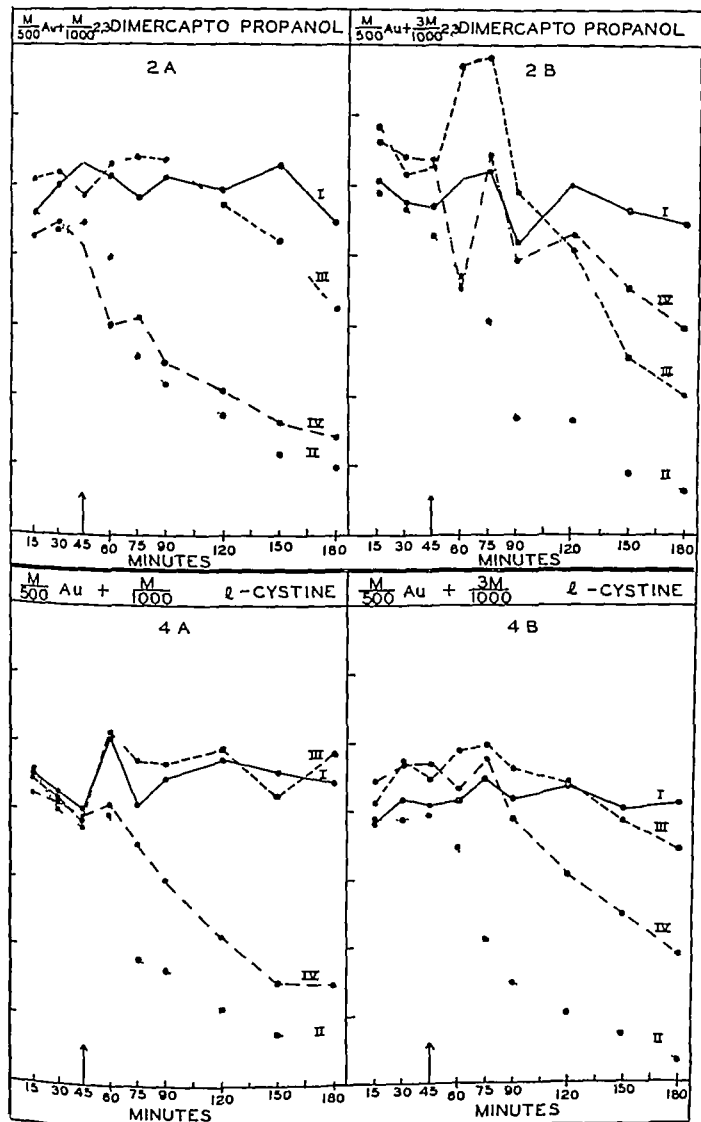


Fig. 2—Continued See legend on opposite page

## DISCUSSION

With the exception of sodium thioglucose, none of the compounds containing a thiol or a potential thiol group definitely affected the inhibition in oxygen consumption caused by gold sodium thiosulfate. Yet all compounds except methionine and cysteine were effective against gold chloride. This could be most clearly demonstrated when three thiol groups were furnished for each atom of gold. However, both BAL and thiomalic acid produced an inhibition of oxygen consumption, when added alone, which was not evident with the other thiol compounds studied. The simultaneous addition of either of these with the gold sodium thiosulfate did not result in an additive effect with respect to respiratory inhibition. When either of these two was added with gold chloride, there was definitely less inhibition than was produced by either the thiol compound or the gold salt alone.

It appears that under conditions of these experiments, there is a chemical reaction *in vitro* between gold chloride and thiol groups to produce a compound which does not inhibit tissue respiration. The gold compounds theoretically formed, for example, gold thiomalate and gold thioglucose, were shown not to inhibit oxygen consumption in previous studies<sup>6</sup>. Postulated compounds formed by the reaction of gold chloride and BAL or L-cysteine would be expected to behave similarly. We have no explanation for the failure of cysteine and methionine to counteract inhibition.

The results with gold sodium thiosulfate are not as clear cut. Because of the complicated chemical nature of this salt, it is difficult to postulate what compounds might be formed by the thiol derivatives.

The ability of thiol groups to remove gold deposited in tissues has not been investigated by this study. The work here presented has shown the ability of thiol compounds to lessen the *in vitro* inhibition of oxygen consumption when the compounds are used simultaneously with certain gold salts.

The implications of these studies with respect to the therapeutic uses of gold are not clear. Gold chloride is extremely toxic and has not been used in treating human beings. Yet it is the only salt which, under the conditions of this study, appears to be detoxified by various thiol groups. The inhibitory action of gold sodium thiosulfate is prevented only by sodium thioglucose, and not by the other thiol groups studied, some detoxifying action by BAL and sodium thiomalate may be masked by the fact that these compounds *per se* inhibit oxygen consumption.

The gold salts commonly used in the treatment of rheumatoid arthritis, gold sodium thiomalate and gold thioglucose, do not interfere *in vitro* with tissue respiration. Since in these two preparations the gold is bound to the organic moiety by a sulfur linkage, it is possible that the empiric development of therapeutic gold preparations has already taken advantage of the thiol detoxifying mechanism. This interpretation cannot be proved by *in vitro* studies, since it is possible that gold salts are metabolized by a common pathway, regardless of the form in which they are introduced into the body. Also, the failure of sodium succinimido-aurate, which contains no sulfur, to interfere with tissue respiration<sup>6</sup> suggests that factors other than sulfur linkage may be important in determining toxicity.

## SUMMARY

1 The *in vitro* inhibition of oxygen consumption of rat kidney slices observed with gold sodium thiosulfate is not lessened by the potential thiol compounds cystine and methionine, nor by BAL or thiomalic acid which contain thiol groups, however, sodium thioglucose does lessen the inhibition of oxygen consumption. The effects of BAL and thiomalic acid may be masked by the fact that these compounds, in themselves produce inhibition of respiration.

2 The *in vitro* inhibition of oxygen consumption of rat kidney slices caused by gold chloride is appreciably reduced by thiomalic acid, BAL, L-cystine and sodium thioglucose, but not by methionine or cysteine.

3 When three thiol groups are furnished for each atom of gold the reduction of inhibition is more clearly shown than when only one thiol group per atom of gold is present.

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The oral hippuric acid liver function test was carried out in the manner described by Quick and commonly used in clinical medicine. As in the foregoing procedures, when para-aminobenzoic acid was used, its administration was started eighteen hours before the ingestion of sodium benzoate and it was given every three hours. When glycine was used, 20 Gm were given with breakfast and 20 Gm with the sodium benzoate.

### RESULTS

When three subjects of similar height and weight were given orally a single 3 Gm dose of sodium salicylate, the plasma salicylate level reached its peak of 18 to 22 mg per 100 cc in two to four hours and then fell rapidly to 1 mg or less per 100 cc in twenty-eight to thirty-two hours. However, when the procedure was repeated and para-aminobenzoic acid was also given, the plasma salicylate level again reached its peak in two to four hours but fell more slowly. In one subject the plasma salicylate level dropped to 1 mg per 100 cc in forty-eight hours, and in the other two subjects it was more than 3 mg per 100 cc at fifty-six hours. Fig 1 shows results obtained on Subject 4 plotted on arithmetic graph paper and Fig 2 shows the same results plotted on semilogarithmic paper. Oral administration of para-aminobenzoic acid appeared to cause the plasma salicylate levels to fall more slowly and to alter the form of the curve.

Table I shows the salicyl fractions found in the urine of Subject 4 in the foregoing study. In man, after oral administration of the single dose of salicylate, there were the expected large quantities of salicyluric acid present in the urine. However, when para-aminobenzoic acid was given with the salicylate only very small quantities of salicyluric acid were found in the urine in spite of collection of the urine for a period almost twice as long. The quantities of free salicylate and salicyl glucuronate were comparable when the duration of the collection period was considered. There was no retention of salicyluric acid in the blood when it failed to appear in the urine, as the plasma salicylate was practically all in the form of free salicylate and only the usual traces of salicyluric acid were present.

TABLE I THE SALICYL FRACTIONS RECOVERED FROM THE URINE OF SUBJECT 4 AND DOG 261 IN THE SAME STUDIES ILLUSTRATED BY FIGS 1 THROUGH 6

	COLLECTING PERIOD (HR)	SODIUM SALICYLATE	SALICYL FRACTIONS IN THE URINE (MG)			
			SALICYLURIC ACID	FREE SALICYLATE	SALICYL GLUCURONATES	TOTAL SALICYLATE
Subject 4	34	Without PAB	1,364.36	319.43	298.78	1,982.57
	60	With PAB	71.50	819.39	610.61	1,501.50
Dog 261	48	Without PAB	4.54	185.46	203.95	393.95
	48	With PAB	0	198.0	264.0	462.0

Four similar studies were done on two dogs, the salicylate was given orally twice and intravenously twice. The results on one dog are shown in Figs 3, 4, 5, and 6. Salicylate excretion was much slower in the dog than in man and when para-aminobenzoic acid was given, the rate of excretion of salicylate was not altered appreciably.

In the dog the administration of para-aminobenzoic acid did not change the salicyl fractions appearing in the urine. Only traces of salicyluric acid were

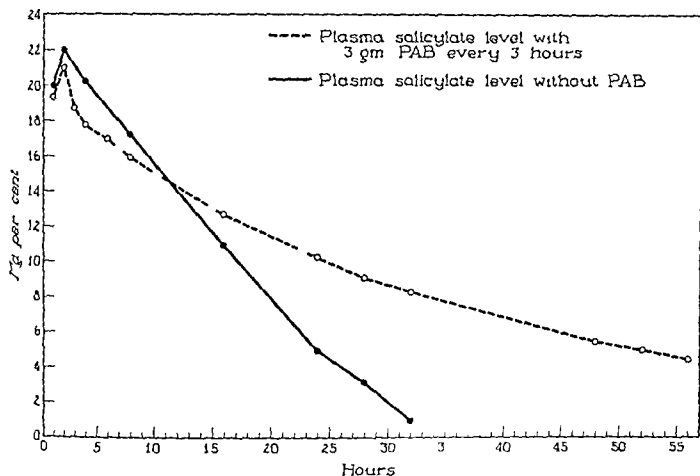


Fig 1—Salicylate concentration in the plasma of Subject 4 after administration of a single dose of 3 Gm of sodium salicylate. Three grams of para aminobenzoic acid were given every three hours throughout the experiment.

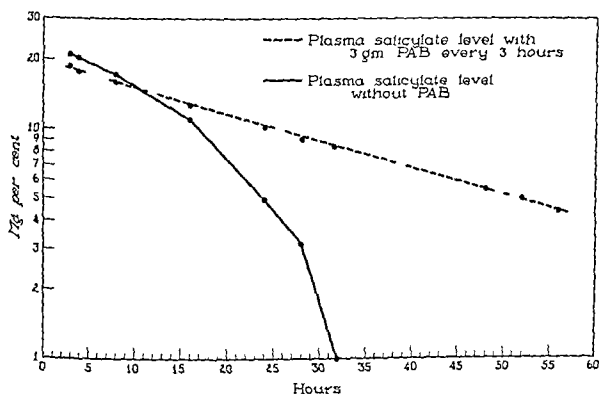


Fig 2—The same values as in Fig 1 plotted on semilogarithmic paper.

present in the urine when salicylate alone was given. Table I shows the salicyl fractions found in the urine of Dog 261 in the foregoing studies.

Table II shows the renal clearance figures obtained on four human subjects. The clearance values of salicylate without para aminobenzoic acid ranged from 50.46 to 99.36 c.c. of plasma cleared per minute, with an average of 70.29 cubic

centimeters The clearance values of salicylate when para aminobenzoic acid was given ranged from 22.76 to 32.41 cc of plasma cleared per minute, with an average of 27.53 cubic centimeters The renal clearance of salicylate apparent

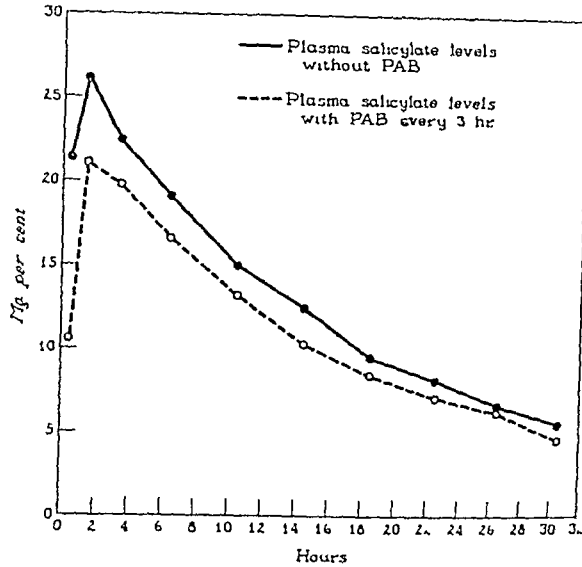


Fig 3—Salicylate concentration in the plasma of Dog 261 after the oral administration of 1 Gm of sodium salicylate. One-half gram of para aminobenzoic acid was given every three hours throughout the study

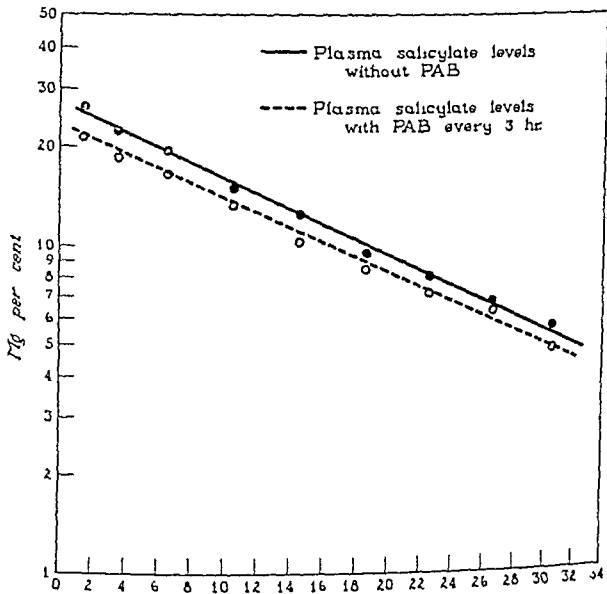


Fig 4—The values in Fig 3 plotted on semilogarithmic paper

was reduced approximately 60 per cent when para-aminobenzoic acid was given. However, in calculating these clearance values the urine was hydrolyzed and the figure for the total salicylate present was used regardless of what salicyl

fractions were present. Since this clearance value is actually the resultant of all the clearance figures of the individual salicyl fractions appearing in the urine, not much information can be obtained from it.

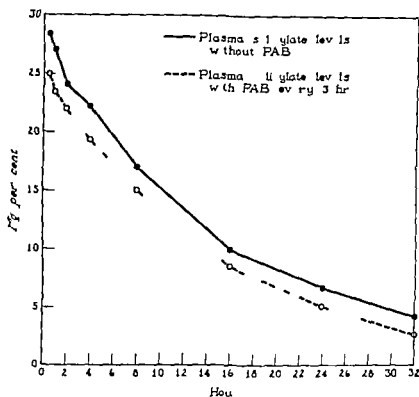


Fig. 5—Salicylate concentration in the plasma of Dog 61 after the intravenous administration of 0.1 Gm. of sodium salicylate per kilogram of body weight. One half gram of para aminobenzoic acid was given every three hours throughout the study.

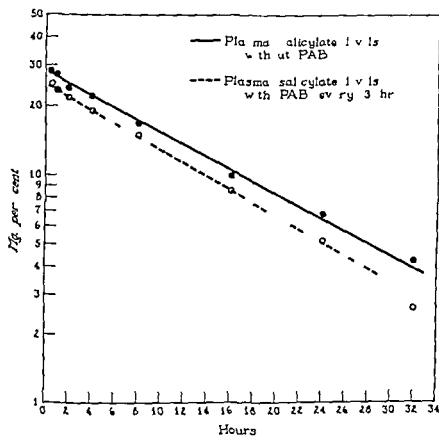


Fig. 6—The values of Fig. 5 plotted on semilogarithmic paper.

Table III shows the results of another group of clearances in which the salicyl fractions in the urine were determined. When para aminobenzoic acid was given with the salicylate again only traces or very small quantities of

TABLE II DECREASE OF THE RENAL CLEARANCE OF TOTAL SALICYLATE AFTER THE INGESTION OF PARA AMINO BENZOIC ACID IN MAN, CLEARANCES ARE GIVEN AS CUBIC CENTIMETERS OF PLASMA CLEARED PER MINUTE

SUBJECT	VOLUME OF URINE (C C PEP MIN)	TOTAL SALICYLATE CLEARANCE (C C PEP MIN)		PLASMA PAB LEVELS (MG PER 100 C C)
		NO PAB	WITH PAB	
1	3 18	77 07		
	1 12	68 70		
	2 70		31 94	12 55
	1 80		32 41	13 23
2	1 65	99 36		
	1 24	73 57		
	2 31		25 41	9 15
	1 26		26 52	8 25
3	1 86	50 46		
	1 56	51 97		
	3 60		22 76	11 14
	1 40		23 13	8 55
4	3 40	77 42		
	1 40	63 75		
	2 75		28 40	10 2
	1 50		29 65	8 6
Average		70 29	27 53	

salicyluric acid appeared in the urine. Since salicyluric acid has a higher renal clearance value than free salicylate and normally constitutes approximately 50 per cent of the total salicyl fractions excreted in the urine, the deficiency of this salicyl fraction caused a marked drop in the renal clearance of the total salicylate. At the same time clearance of the free salicylate fraction remained essentially unchanged unless the pH of the urine was also decreased. However, when enough sodium bicarbonate was given with the salicylate and para aminobenzoic acid to produce a strongly alkaline urine, the clearance of the free salicylate fraction increased enough to mask completely the effect of the deficiency of

TABLE III THE EFFECTS OF THE INGESTION OF PARA AMINO BENZOIC ACID, GLYCINE, AND SODIUM BICARBONATE ON THE EXCRETION AND CLEARANCE OF THE SALICYL FRACTIONS IN MAN

SUBJECT		VOLUME OF URINE (C C PEP MIN)	PH OF URINE	SALICYL FRACTIONS IN THE URINE (MG IN 1 HR)				RENAL CLEARANCE (C C OF PLASMA PER MIN)	
				SALI CYL URIC ACID	FREE SALI CYL ATE	SALICYL GLU CUROV ATE	TOTAL SALI CYL ATE	FREE SALI CYL ATE	TOTAL SALI CYL ATE
1	No PAB	1 8	6 8	72 46	30 0	32 61	135 07	17 68	79 93
	With PAB	2 27	6 4	8 31	25 85	34 28	68 44	12 11	32 00
	With PAB plus glycine	2 73	7 5	5 12	117 19	30 38	152 69	61 03	79 30
2	No PAB	1 88	6 9	60 00	32 42	38 64	131 06	19 64	79 42
	With PAB	1 60	6 85	16 94	41 66	40 85	99 45	19 44	46 4
	With PAB plus sodium bicarbonate	2 70	8 0	Trace	197 50	32 5	230 0	106 93	124 0
3	With PAB plus sodium bicarbonate	1 20	7 85	12 4	159 36	33 5	205 26	92 96	119 75
	With sodium bicarbonate	1 75	7 75	68 45	162 25	38 4	269 10	98 33	163 1
4	With PAB plus glycine	2 07	7 6	5 0	114 80	50 65	170 45	62 62	92 99



saheyluric acid on the total salicylate clearance. In an attempt to see if the quantity of available glycine was the limiting factor, glycine was given with the para aminobenzoic acid and salicylate but still only small quantities of saheyluric acid were found in the urine. The urine was consistently alkaline when glycine was ingested and the excretion of total salicylate was high because of the increased renal clearance of the free salicylate fraction.

TABLE IV. DECREASE OF THE FORMATION OF HIPPURIC ACID IN MAN AFTER THE INGESTION OF PARA AMINOBENZOIC ACID AS INDICATED BY THE ORAL HIPPURIC ACID LIVER FUNCTION TEST

SUBJECT	DAY (JUNE 1947)	GRAMS OF HIPPURIC ACID EXCRETED IN THE URINE IN FOUR HOURS AFTER ADMINISTRATION OF		
		6 GM SODIUM BENZOATE WITH PAB	6 GM SODIUM BENZOATE WITHOUT PAB	6 GM SODIUM BENZOATE WITH PAB PLUS GLYCINE
1	25	0.398	4.980	0.650
	28			
	30			
2	25	0.542	4.43	0.400
	28			
	30			
3	25			0.485
	30			

In order to see if para aminobenzoic acid was capable of interfering with the conjugation of glycine with benzoic acid or its derivatives other than salicylic acid the hippuric acid liver function test was used. Table IV shows that there was a great decrease of the formation of hippuric acid when para amino benzoic acid was given and the exogenous glycine did not eliminate this effect.

#### COMMENT

The results of this study substantiate the finding of Dry, Butt, and Scheinley that the oral administration of para aminobenzoic acid caused an elevation of the plasma salicylate level in man. This elevation did not occur in the dog. The mechanism responsible for this elevation of plasma salicylate level in man appeared to be an alteration of the detoxication of salicylate.

According to the reports of Bertagnini,<sup>7</sup> Tollens,<sup>8</sup> Quick,<sup>9</sup> Salt,<sup>10</sup> Kapp and Coburn,<sup>11</sup> and Smith and co workers,<sup>4</sup> man normally conjugates salicylic acid with both glycine and glucuronic acid and the corresponding products saheyluric acid and saheyl glucuronates appear in the urine. Kapp and Coburn reported that approximately 80 per cent of the ingested salicylate was excreted in the urine as compounds containing the salicyl radicle and roughly 50 per cent of this was saheyluric acid. This conjugation probably occurs in the liver. Quick<sup>12, 13</sup> has pointed out that the liver of the dog does not conjugate glycine with benzoic acid and its derivatives to the same extent as the liver of man and many animals, but does use glucuronic acid extensively in the conjugation of benzoic acid and orthohydroxybenzoic acid.

Working with rabbits Ellinger and Hensel<sup>14, 15</sup> in 1914 first reported the excretion of acetyl para aminobenzoic acid after the feeding of para amino benzoic acid. After this report, para aminobenzoic acid was widely used in the study of some of the mechanisms of detoxication in man as well as animals, and

the literature on the metabolism of the drug is extensive<sup>10, 21</sup>. These reports indicate that, in man, para-aminobenzoic acid is conjugated with glycine and glucuronic acid and that the corresponding products appear in the urine in addition to acetyl-para-aminobenzoic acid.

The findings in this study are in agreement with the foregoing observations in that only traces of salicylic acid were found in the urine of the dogs after the administration of salicylate, while in man large quantities of salicylic acid were excreted in the urine after the ingestion of salicylate. When para-aminobenzoic acid was given to man with sodium salicylate, only traces or very small quantities of salicylic acid appeared in the urine. Since there was no retention of salicylic acid in the blood when it failed to appear in the urine, its formation was apparently interrupted or markedly decreased. Although the principal mechanism by which para-aminobenzoic acid increased the plasma salicylate level and decreased total salicylate excretion appeared to be the interruption of salicylic acid formation, para-aminobenzoic acid also tended to lower the pH of the urine and thus further decreased the total salicylate excretion by decreasing the renal clearance of the free salicylate fraction. When the urine was made strongly alkaline by the administration of massive doses of sodium bicarbonate, the clearance of the free salicylate fraction increased, as observed by Smith and co-workers,<sup>4</sup> and the effect of para-aminobenzoic acid was masked.

Because of the fact that no relatively simple methods were available for determining the concentrations of the conjugated products of para-aminobenzoic acid in the urine or blood, no attempts were made to study the possible reciprocal effects of salicylic acid on the detoxication or excretion of para-aminobenzoic acid.

#### SUMMARY

The oral administration of para-aminobenzoic acid appeared to have the following effects on the metabolism and excretion of salicylate in man. First, it altered the detoxication of salicylate by interrupting or greatly depressing the conjugation of glycine with salicylate so that only very small quantities of salicylic acid appeared in the urine after ingestion of salicylate. Second, it tended to lower the pH of the urine and thus decreased the renal clearance of the free salicylate fraction. Third, it caused a decrease of the urinary excretion of total salicylate and a rise of the plasma salicylate level due to the foregoing effects.

In dogs the administration of para-aminobenzoic acid did not alter the excretion of salicylate and did not increase the plasma salicylate levels.

The administration of para-aminobenzoic acid appeared to decrease greatly the formation of hippuric acid as measured by the oral hippuric acid liver function test in man.

These effects were temporary and completely reversible.

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## USE OF PENICILLIN AND STREPTOMYCIN IN THE ISOLATION OF POLIOMYELITIS VIRUS FROM FECAL SPECIMENS

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IN ORDER to help confirm the diagnosis of poliomyelitis that had been made in several epidemics during the past two years, it was necessary to recover the virus from fecal specimens. Melnick<sup>1</sup> has recommended methods of preparation by high-speed centrifugation, but because the instruments were not available, the etherization method of Paul and Trask<sup>2</sup> was used. Isolations of the virus were made in two epidemics but because so often the monkeys succumbed to peritonitis after several intra-abdominal inoculations, another method was sought to reduce the bacterial content without loss of virus activity.

It had been shown by various workers that the antibiotics penicillin and streptomycin have little effect on the virulence of certain viruses, while on the other hand they greatly reduce the associated bacteria. Parker and Diefendorf<sup>3</sup> reported that the viruses of vaccinia, St. Louis encephalitis, and of equine encephalomyelitis were able to grow in the chicken embryo after addition of penicillin. Floirnan, Weiss, and Council<sup>4</sup> had used streptomycin without any effect against influenza A virus, Hirst<sup>5</sup> likewise found penicillin to be of use in the isolation of the latter virus, while Hodges<sup>6</sup> obtained marked reduction of bacteria after the simultaneous inoculation of penicillin, streptomycin, and fecal specimen into eggs. Bradley and co-workers<sup>7</sup> employed penicillin to inhibit bacterial growth in the isolation of Newcastle disease virus from contaminated chicken tissues, while Beaudette, Bruns, and Miller<sup>8</sup> have recently advocated the use of a mixture of penicillin and streptomycin for the recovery of the latter virus from chickens. Because the organisms were inhibited and yet none of the viruses used were particularly affected by these antibiotics, the following studies were undertaken with the virus of poliomyelitis. Before testing the antibiotics directly on fecal specimens, it seemed advisable to determine both what effect penicillin and streptomycin might have on different dilutions of poliomyelitis virus and what might be the optimal concentrations for inhibiting bacterial growth.

*Experiment 1 To Determine the Effect of Streptomycin and Penicillin on Different Dilutions of the MV Virus of Poliomyelitis*—A 10 per cent aqueous suspension of monkey cord containing the MV† monkey passage virus of poliomyelitis was centrifugated at 6,000 revolutions per minute for thirty minutes in the angle centrifuge placed in the cold room. The supernatant fluid was diluted

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TABLE I EFFECT OF PENICILLIN AND STREPTOMYCIN ON MV POLIOMYELITIS VIRUS ALONE AND ON A MIXTURE OF VIRUS AND BACTERIA

EXPERIMENT	RHESUS MONKEY	MATERIAL INOCULATED	ROUTE OF INOCULATION*	DILUTION OF VIRUS	RESULTS
1	M 148	MV virus and anti biotics	in and i abd	1 10	Paralyzed
	M 149	MV virus and anti biotics	in and i abd	1 100	Paralyzed
	M 116	MV virus and anti biotics	in and i abd	1 500	Paralyzed
	M 147	MV virus alone	ic	1 10	Paralyzed
2	M 121	MV virus fly sus pension, and anti biotics	in and i abd	1 10	No symptoms With stood challenge dose of virus
	M 122	MV virus fly sus pension and anti biotics	in and i abd	1 100	Paralyzed
	M 123	MV virus fly sus pension and anti biotics	in and i abd	1 1000	Paralyzed
	M 124	MV virus fly sus pension and anti biotics	in and i abd	1 10 000	No symptoms With stood challenge dose of virus
	M 150	MV virus fly sus pension and anti biotics	ic	1 10	Paralyzed
	M 151	MV virus alone	ic	1 10	Paralyzed
	M 137	MV virus normal feces and anti biotics	in and i p	1 10	Paralyzed
3	M 138	MV virus normal feces and anti biotics	in and i p	1 100	No symptoms
	M 139	MV virus normal feces and anti biotics	in and i p	1 1000	No symptoms With stood challenge dose of virus
	M 136	MV virus normal feces and anti biotics	ic	1 10	Paralyzed
	M 152	MV virus alone	ic	1 10	Paralyzed

\* in Intranasal instillation total of 15 ml i abd intra abdominal inoculation total of 30 ml ic. Intracerebral inoculation of 1 milliliter

1 100 and 1 500 and 15 ml of each dilution were frozen for future use. The antibiotics were then added to the remainder of the three dilutions (1 10 1 100 and 1 500) in such a concentration that each contained 1 250 units of penicillin and 25 mg of streptomycin per milliliter. After remaining one half hour at room temperature, Rhesus monkeys were inoculated intra abdominally with 16 ml of each treated dilution of virus and intranasally with 3 ml of each that remained untreated. Daily injections were made until a total of 32 ml of each material was given by the first route and 15 ml by the second. One milliliter of the 10 per cent suspension of MV virus was given intracerebrally to a fourth monkey as a control. All of the animals became completely paralyzed as shown in Table I Experiment 1. It was apparent that the antibiotics in these concentrations did not reduce the potency of MV virus diluted at least 1 500. In fact the results were more effective than when this same lot of MV virus was titrated in monkeys without either material. With intracerebral inoculations of 1 ml of each tenfold dilution from  $10^{-1}$  through  $10^{-5}$ , only the monkey receiving the first

dilution became completely paralyzed. However, when given larger amounts by the combined intranasal and intra-abdominal routes, both the animals receiving the  $10^{-1}$  and the  $10^{-3}$  dilutions showed typical paralysis.

*Experiment 2 To Determine the Effect of Penicillin and Streptomycin on a Mixture of MV Poliomyelitis Virus and Bacteria Found in Flies*—To test the effect of the antibiotics on different virus concentrations in the presence of bacteria, tenfold dilutions of the MV strain were made in a suspension of flies that had been taken at the city dump. After centrifugation for thirty minutes at 6,000 revolutions per minute, each dilution was treated with the same concentrations of the antibiotics as used in Experiment 1. Likewise, Rhesus monkeys were inoculated with the same amounts of material given by the same routes as in Experiment 1. At the same time 1 ml. of the treated  $1/10$  suspension was inoculated into a monkey intracerebrally, while another animal was given a similar dose of the untreated  $1/10$  virus by the same route.

All but two of the monkeys became paralyzed as shown in Table I, Experiment 2. These two probably had an inapparent infection because both withstood a paralyzing dose inoculated intracerebrally at a later date. Likewise, it was of interest that the monkey (M-150) receiving an intracerebral injection of the treated fly material became typically paralyzed without developing a brain abscess.

*Experiment 3 To Determine the Effect of Penicillin and Streptomycin on a Mixture of MV Virus and the Bacteria of a Normal Fecal Suspension*—A 20 per cent suspension of normal feces was made in sterile distilled water. Tenfold dilutions of MV virus were prepared in this material and after a light centrifugation a portion of each dilution was frozen for future use. The remaining dilutions were run in the angle centrifuge for thirty minutes at 13,000 revolutions per minute. Sufficient of each dilution was prepared so that aliquot portions could be frozen and removed when needed. One half hour before the inoculations a 16 ml. vial of each dilution,  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$ , was thawed and the antibiotics quickly were added in an amount so that each dilution of virus contained 1,250 units of penicillin and 25 mg. of streptomycin. Rhesus monkeys were given five daily doses of 3 ml. of the untreated material intranasally and two daily doses of 16 ml. each of the treated dilutions intra-abdominally. One animal was injected with 1 ml. of the  $1/10$  treated material intracerebrally.

As may be seen in Table I, Experiment 3, the virus diluted with fecal suspension was not as potent as that with the fly material. No paralysis occurred beyond the  $1/10$  dilution. The same lot of frozen virus was used in both experiments. However, once again typical paralysis was produced by the intracerebral route without any evidence of bacterial contamination.

*Experiment 4 Effects of the Antibiotics on Fecal Bacteria*—The following experiments were made to determine the approximate optimal bacteriostatic concentration of the antibiotics which could be used with a 20 per cent fecal suspension.

(1) Penicillin (crystalline sodium G) and streptomycin (calcium chloride complex, Meck) were added to a 20 per cent normal fecal suspension in the concentrations shown in Table II. In one series the antibiotics had little effect upon

TABLE II BACTERIOSTATIC EFFECT OF PENICILLIN AND STREPTOMYCIN ON A FECAL SUSPENSION

30 MIN AT ROOM TEMPERATURE		CULTURES			
AMOUNT PER ML.		SUSPENSION UNCENTRIFUGED		SUSPENSION CENTRIFUGED 30 MIN AT 13,000 R.P.M.	
PENICILLIN (UNITS)	STREPTOMYCIN (MG.)	BLOOD AGAR	BEEF HEART BROTH	BLOOD AGAR	BEEF HEART BROTH
416.6	6.25	Gr - rods* Hem strep	Cr + rods	0	have Gr + rods
390.2	5.90				
60.0	12.50	Gr - rods Hem strep	Gr + and Cr - rods	0	0
1000.0	20.00				
1000.0	20.00	Gr - rods Hem strep	Cr + and Cr - rods	0	0
1000.0	20.00			0	0
1190.0	11.90	Gr - rods Hem strep	Cr + and Cr - rods	0	0
1111.0	22.20				
2000.0	40.00	Gr - rods Hem strep		0	0
2100.26	52.60			0	0 - Transfer on blood agar Cr + rods
2381.0	23.80	Gr - rods Hem strep			
None	None	Much growth	Gr + and Gr - rods	Slight growth	Not inoculated

Gr - Gram negative  
Gr+ Gram positive

the aerobic or anaerobic growth of the bacteria on blood agar and in beef heart broth, respectively, when the suspension was not sufficiently centrifugated while in the other the results were satisfactory when the material was run in the angle centrifuge for thirty minutes at 13,000 revolutions per minute

TABLE III COMPARISON OF THE BACTERIOSTATIC EFFECTS OF ETHER AND OF ANTIBIOTICS ON A FECAL SUSPENSION

30 MIN AT ROOM TEMPERATURE		CULTURES		ETHERIZATION MET IOD	CULTURES	
AMOUNT PER ML. FECAL SUSPENSION		BLOOD AGAR	THIOGLYCOLATE BROTH		BLOOD AGAR	THIOGLYCOLATE BROTH
PENICILLIN (UNITS)	STREPTOMYCIN (MG.)					
100	0	0	0	10% ether	0	Gr - rods
200	4.0	0	0	10% ether	0	Gr - rods Gr + cocci
300	6.0	0	0			
400	8.0	0	0			
500	10.0	0	0			
600	12.0	0	0			
700	14.0	0	0			
800	16.0	0	Few Cr + and Gr - rods			
900	19.0	0	0			
1000	20.0	0	0			
None	None	0	Gr - rods Gr + rods and cocci	No ether	0	Gr - rods Gr + rods and cocci

(2) Also, a comparison was made of the bacteriostatic effect of etherization with that of the antibiotics. A 20 per cent suspension of normal feces in sterile distilled water was centrifugated for ten minutes at 2,000 revolutions per minute to throw down the coarse particles. The supernatant fluid was removed and run in the angle centrifuge for thirty minutes at 13,000 revolutions per minute. Cultures were made on blood agar and in thioglycolate broth. The material was divided and one part was treated with 15 per cent ether and the other with varying concentrations of the two antibiotics. The etherized portion was shaken for thirty minutes in the cold room and left overnight, the ether was removed by suction the next morning. Cultures were made in the media mentioned.

As may be noted in Table III, the ether attenuated the development of aerobic bacteria on blood agar but did not reduce growth in the anaerobic liquid medium. On the other hand, organisms were inhibited on both types of media when practically all of the antibiotic concentrations were used.

From the results both of titrations in monkeys and of the cultural tests, it seemed evident that the use of penicillin and streptomycin would be advantageous in the isolation of the poliomyelitis virus from fecal specimens. The optimal concentrations for bacterially infected material appeared to be 1,000 units of penicillin and 20 mg of streptomycin per milliliter. These amounts retarded bacterial growth without reducing the virus potency. Therefore the method seemed sufficiently reliable for use on human fecal material.

*Experiment 5 Use of the Antibiotics in the Preparation of Human Feces From Patients With Poliomyelitis—*

*With Known Positive Feces*—Before trying this method on feces of unknown virus content, material was prepared from a human stool that had yielded poliomyelitis virus by the etherization method. This specimen had been kept frozen in the dry ice refrigerator for at least two months. A 20 per cent suspension in sterile distilled water was divided into two portions, one of which was treated with ether by the aforesaid method and the other with the antibiotics essentially in the manner described under Experiment 3, except for the following differences. The dilutions were 1:5, 1:10, 1:50, and 1:100, and 1,000 units of penicillin and 20 mg of streptomycin were used per milliliter of dilution. Cynomolgus monkeys were inoculated throughout the experiment. The different dilutions of feces treated by the two methods were inoculated into the animals intraabdominally. The untreated suspensions were given intranasally.

TABLE IV COMPARISON OF VIRUS ISOLATION METHODS IN CYNOMOLGUS MONKEYS USING A SUSPENSION OF POSITIVE POLIOMYELITIS FECES

METHOD	AMOUNT AND ROUTE OF INOCULATION *	RESULTS			
		DILUTIONS OF POSITIVE POLIOMYELITIS FECES			
		1:5	1:10	1:50	1:100
Etherization	30 ml iabd 15 ml in	0	Paralyzed	0	0
Penicillin and streptomycin	30 ml iabd 15 ml in	Paralyzed	Paralyzed	Paralyzed	0
Penicillin and streptomycin	1 ml ic	Paralyzed	Paralyzed	0	0

Histopathologic sections on all paralyzed monkeys were positive for poliomyelitis.

\*iabd Intra-abdominal in intranasal ic Intracerebral



A third series of monkeys was injected intracerebrally with 1 ml amounts of each dilution of feces treated with the antibiotics. As shown in Table IV, only the monkey given the 1:10 dilution of feces became paralyzed after the etherization method, while the animals given all three dilutions (1:5, 1:10 and 1:50) developed poliomyelitis after employing the antibiotic technique. No animals developed peritonitis nor did any show evidence of a brain abscess when inoculated intracerebrally. It seemed apparent that better results were obtained in the isolation of the virus by the antibiotic method than by that of etherization. The former was also simpler and quicker to prepare.

*With Feces From Patients with Suspected Poliomyelitis*—Fecal specimens obtained during the first week of illness were received from eight patients with suspected poliomyelitis. Each sample was diluted in sufficient sterile distilled water to make a 20 per cent suspension which was then centrifugated for ten minutes at 2,000 revolutions per minute to throw down the larger particles. The supernatant fluid was removed. About 15 ml were saved and frozen in 3 ml amounts to be used for the daily intranasal instillations of *Cynomolgus* monkeys. The remainder of the material was put in the angle centrifuge for thirty minutes at 13,000 revolutions per minute in the cold room (9 to 10° C). The supernatant fluid was then kept frozen in 10 ml amounts until needed.

Thirty minutes before the inoculations were to be made 10 ml of the suspension were thawed rapidly and treated with 1,000 units of buffered crystalline sodium penicillin G and 20 mg of streptomycin (calcium chloride complex, Merck) per milliliter. After leaving the mixture for thirty minutes at room temperature cultures were made on blood agar and in thioglycolate broth. A monkey was then inoculated intra-abdominally with 10 ml of the treated material and intranasally with 3 ml of the untreated feces. The same amounts were repeated daily over a five-day period until a total of 30 ml was given into the peritoneal cavity and 15 ml by the nasal route.

Four of the eight monkeys developed flaccid paralysis typical of poliomyelitis within from six to eight days after the first injection. They were sacrificed and suspensions of the cord were passed intracerebrally to monkeys, guinea pigs, and albino mice. The passage monkeys likewise developed symptoms of poliomyelitis but the other animals remained well. Histopathologic sections of the first passage monkey cord showed lesions of poliomyelitis.

The results of these inoculations again indicated that the additions of penicillin and streptomycin in the quantities employed is a satisfactory aid in the isolation of the virus of poliomyelitis from bacterially contaminated suspensions.

#### DISCUSSION

Although the treating of fecal specimens with ether in the isolation of the poliomyelitis virus has yielded adequate results in determining the causative agent in an outbreak of suspected poliomyelitis, there is always the risk of losing monkeys from peritonitis even if the bacterial counts seem negligible. If the peritoneal route is omitted and one depends solely on the intranasal instillations the quantity of material administered may not be sufficient unless the time period is increased. Therefore it is gratifying to find that one can satisfac-

torily add penicillin and streptomycin in the afore-mentioned amounts to fecal or other bacterially contaminated material suspected of containing the virus of poliomyelitis

In attempted isolation of this virus from feces obtained in an earlier outbreak, five out of twenty-two monkeys (22 per cent) died from peritonitis after intra-abdominal inoculation of etherized specimens. After inaugurating the antibiotic method, no animals developed peritonitis out of the twenty five tested intra-abdominally, and none of the monkeys had a brain abscess after intra-cerebral injection of treated material. In applying this method, however, it is essential that the penicillin and streptomycin should be added to the fecal suspension after clarification in the angle centrifuge at 13,000 revolutions per minute. The virus is not sedimented at that speed and this type of machine may be available in most virus laboratories that do not have an ultracentrifuge. This method greatly reduces the time of preparation because the antibiotics react quickly and it is not necessary to leave the mixture in contact for more than thirty minutes.

As shown in the foregoing experiments (Tables I and IV), there is apparently no loss of virus potency because it may be recovered even after dilution of a fecal specimen 1:50, whereas the results were negative with this dilution when the same sample was treated with ether. There is sufficient evidence to show, therefore, that 1,000 units of penicillin and 20 mg. of streptomycin per milliliter do not materially inhibit the recovery of the poliomyelitis virus from contaminated material even though in a comparatively low concentration.

After this work was completed it was of interest to learn that Melnick<sup>9</sup> had been adding these antibiotics to human feces processed in the ultracentrifuge and that Comia<sup>10</sup> in Cuba also had been adding penicillin to fecal specimens with satisfactory results in the recovery of the poliomyelitis virus.

#### SUMMARY

It has been found that a mixture of 1,000 units of penicillin and 20 mg. of streptomycin per milliliter of material are the optimal concentrations to use for the inhibition of bacterial growth and the isolation of the virus of poliomyelitis from fecal specimens.

A method of isolating the virus from contaminated material has been described which has proved to be quicker and more efficient than the older etherization procedure.

Whereas previously five out of twenty-two (22.7 per cent) monkeys had developed peritonitis after intra-abdominal inoculation by the ether method, none of the twenty-five animals was lost after treatment of the suspensions with the antibiotics, and the same material could be used effectively by the intra-cerebral route as well.

The antibiotics did not prevent paralysis in monkeys when added to a 1:500 dilution of the MV strain, to a 1:1,000 dilution of MV virus and flv suspension, to a 1:10 dilution of MV virus and feces, or to a 1:50 dilution of a known positive fecal specimen.

With the new method, the virus of poliomyelitis was isolated from four out of eight (50 per cent) human feces taken during a recent outbreak of poliomyelitis.

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## THE EFFECT OF COMMERCIAL HEPARIN ON THE PLATELET COUNT

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COPLEY and Robb<sup>1</sup> observed that when heparin was added to dog's blood *in vitro*, it failed to preserve the blood platelets. This was in contrast to the action of citrate which is presumed to preserve platelets because of its anticoagulant properties. The platelet count was lowered sharply within five minutes and after twenty-four hours showed reductions of from 30 to 100 per cent. Since the effect of heparin increased with its concentration and since Copley and Robb<sup>1</sup> also found that intravenous injections depressed the platelet count, there was indication of a direct action rather than a simple failure to preserve the platelets from unfavorable conditions *ex vivo*. This observation is of importance in considering the effect of heparin in the prevention of platelet thrombi. In the course of other studies on heparin, we have been able to confirm and extend the observations of Copley and Robb, and in a personal communication from Dr A J Quick we learn that our results on dogs agree in general with some recent findings at his laboratory.\*

### METHODS

Platelet counts were made from blood taken into Rees and Ecker's formalin citrate mixture recommended by Tocantins<sup>2</sup> and the technique of counting was in some cases modified by the use of 5 per cent urea solution as described by Fidler and Waters<sup>3</sup> so that estimates of leucocytes could also be made in the same chamber in which the platelets were counted. Agglutination was estimated by counting clumps of three or more separately and expressing as a percentage of the total platelet count. In studying the influence of heparin on platelets outside the body, the silicone technique was used as described by Jaques, Fidler, Feldt and Macdonald<sup>4</sup> in order to minimize the effect of foreign surfaces. Concentration of heparin is expressed in Connaught anticoagulant units. This is the activity of 1/100 mg of the crystalline barium salt or 1/110 mg of the sodium salt of beef heparin.

### RESULTS

In a series of experiments designed to study the physiology of heparin, various doses of the anticoagulant were injected into dogs. The effect of these injections on red cell, white cell, and platelet counts is shown in Table I. Attention is directed to the following points:

- (1) A fall in the platelet count occurred in all instances and in four reached a level of less than 10 per cent of the number before injection.
- (2) Fifty units per kilogram had as marked an effect in one animal as 1,500 units per kilogram had in another, suggesting either individual variability or no

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\*See p 1424.

†All the heparin was supplied by the Connaught Medical Research Laboratories, Toronto, Ont., except one sample of Danish heparin obtained through the kindness of Dr Tage Astrup.

correlation between dosage and the extent of effect provided the dose was beyond some minimum and given in a single injection

(3) Agglutination of the platelets appeared in all samples taken two and one-half minutes to more than one hour after injection but clumping under ten per cent was not regarded as significant

(4) After single injections the count would recover from the initial fall but continuous injection would hold the count down although not at as low a level as followed the first injection

(5) On the whole, the leucocytes declined and returned parallel with the platelets after single injections but remained at a lower level with continuous injection

TABLE I CELL COUNTS AFTER INTRAVENOUS INJECTION OF HEPARIN IN THE DOG

EXPERIMENT	HEPARIN (UNITS/KG)	TIME	P B C / C MM × 1 000	W B ( C MM 1 (100)	PLATELETS / C MM	
					× 1 000	AGGLUTINATION (%)
22	500	Before	5 586	12 1	495	0
		3 1/4 min	5 594	12	135	56
3	500	Before	4 763	6 4	366	0
		1 1/2 min	4 956	8 0	143	59
		1 1/2 min	4 781	2 6	148	12
5	500	Before	5 206	8 9	316	0
		15 min	5 213	6 6	209	10
24	100	Before	5 794	10 0	295	1
		5 min	5 781	5 2	20	24
		7 min	5 981	9 1	228	5
25	1500	Before	5 369	8 5	341	0
		3 1/2 min	5 363	1 8	15	27
		60 min	6 013	3 8	115	42
30	50	Before	4 225	9 7	295	0
		Then by cont inj	3 456	5 9	21	67
		80 min	4 251	3 1	107	60
7	50	Before	6 306	9 8	397	0
		4 min	6 344	3 8	39	48
		Then by cont inj *	5 655	3 8	234	27
		65 min	6 881	14 0	203	21
		407 min	6 400	25 0	191	0
9	30	17 1/4 hr after cont inj ceased				
		Before	6 500	10 1	356	0
		4 min	6 256	12 5	178	32
		30 min	6 094	13 0	298	2
		Before	4 500		90	0
16 1A	1 000 u/kg injected in 1 sec	2 min	4 500		50	0
		5 min	4 700		50	20
		10 min	4 500		50	50
		60 min	4 600		85	0
		Before	5 400		120	0
16-2	1 000 u/kg injected in 1 sec	3 min	5 000		30	0
		10 min	5 000		115	0
		60 min	5 400		130	0
		Before	5 000		142 5	0
		2 min	4 800		50	0
16 1B	1 000 u/kg injected in 1 min	5 min	5 000		55	0
		10 min	5 100		60	0
		30 min	4 500		85	0
		60 min	5 100		120	0
		Before	5 100		120	0

Continuous injection of 1 unit per kilogram per minute

In the first eight experiments platelet counts were made in the 1/10 dilution with urea.

In the last three experiments platelet counts were made in the 1/100 dilution

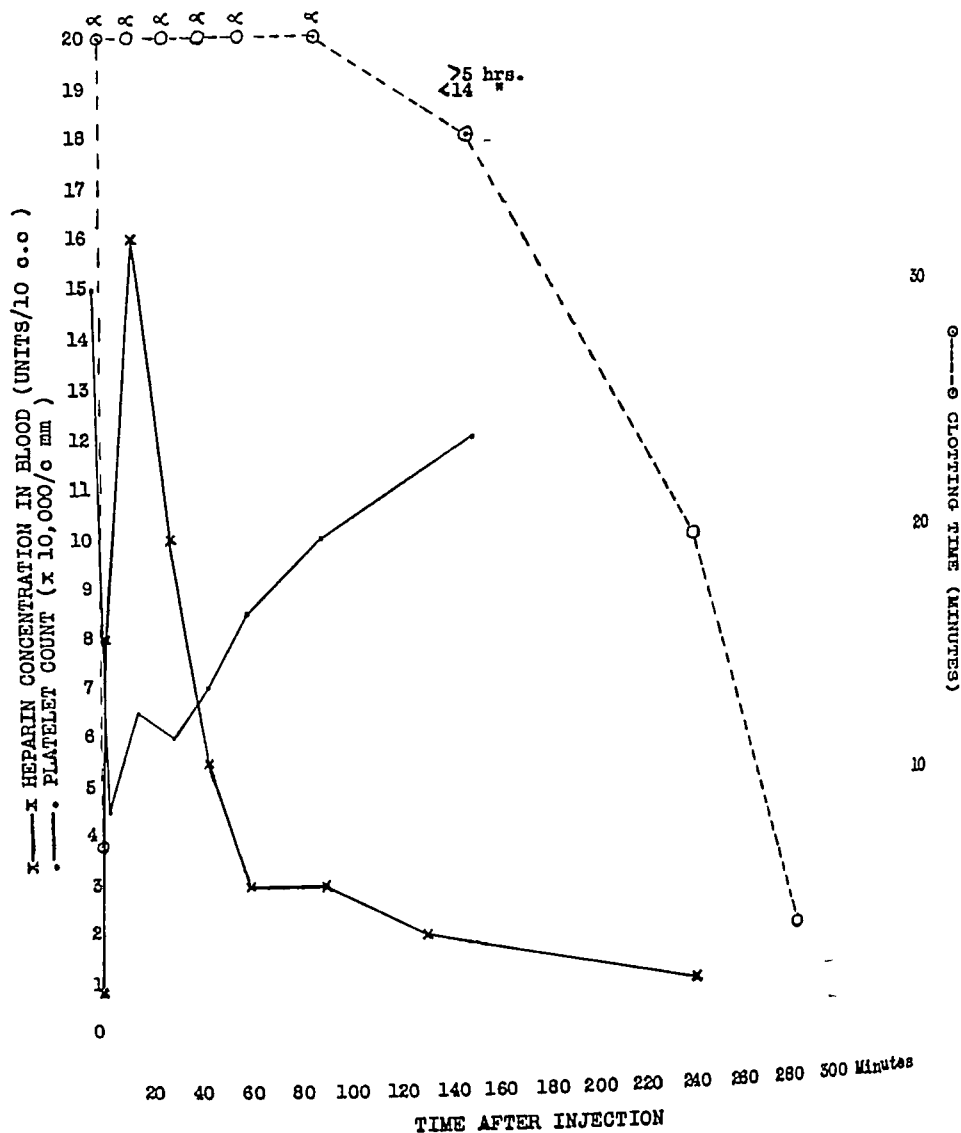


Fig. 1

The effects of continuous injection suggested that as long as heparin remained in the blood stream the counts would remain depressed. This was given further investigation in an experiment shown in Fig. 1 in which heparin concentration in the blood was determined by the method of Jaques, Monkhouse, and Stewart<sup>6</sup>. Four milligrams per kilogram of heparin were injected intravenously into a dog weighing 6.7 kilograms under pentobarbital anesthesia. Clotting times were determined by the method of Lee and White,<sup>7</sup> using blood from the exposed femoral vein. After injection of the heparin the platelet count fell to one-third of the initial value and then rose again, moving inversely with the heparin in the blood. It will be noted, however, that when the platelets recovered to 80 per cent of the original count, there was still enough heparin present

TABLE II EFFECT OF INJECTED HEPARIN ON THE PLATELET COUNT IN L B J

HEP PARIN (LOT 1123)	TIME FROM INJECTION	R.B.C./C MM × 1 000	PLATELETS/C MM × 1 000
40 units/kg intravenously	Before		255
	45 sec		190
	7 min		155
	30 min		255
40 units/kg subcutaneously	Before	5 000	180
	5 min	5 250	190
	1 hr	4 100	125
	2 1/2 hr	4 900	90
	5 hr	5 000	110
	23 hr	5 050	150
	47 hr	9 1	185

Counts made in the 1/100 dilution

to prolong the clotting time to more than five hours. This extension of the anti-coagulant action beyond the effect upon platelets will be discussed later.

Table II shows the effect of heparin upon one of us (L B J) when administered both intravenously and subcutaneously. The latter route merely delayed and prolonged the thrombocytopenia.

Copley and Robb<sup>1</sup> showed that when mixtures of heparin and blood were stored *in vitro*, the greater part of the fall in the platelet count took place in the first forty or fifty minutes when compared with counts obtained at forty eight hours. In the hope of securing further information we made observations on blood handled by means of the silicone technique. Under proper conditions and without the addition of any anticoagulant it is sometimes possible by this method to obtain blood in which the platelet count remains practically unaltered for thirty minutes. Blood removed with a silicone syringe was transferred to small beakers coated with silicone and containing varying quantities of heparin. Since the controls with saline alone could not be used beyond thirty minutes controls in citrate were used in some instances. For storage the mixture was poured into a silicone test tube stoppered and placed in the refrigerator. In a few experiments a further control blood was also placed in a beaker without a silicone surface.

TABLE III BLOOD AND HEPARIN MIXED IN SILICONE VESSELS

PLATELET COUNTS IN FIRST SAMPLE AFTER MIXING				
SUBJECT	CONTROL MIXTURE		HEPARIN MIXTURE	
		PLATELETS/C MM × 1 000		UNIT/C C
Man	Saline	220	140	10
	Saline	190	125	10
Dog	Saline	135	120	100
	Citrate	312	220	0.1
	Citrate	312	19	1.0
	Citrate	312	219	10
	Citrate	312	232	100
	Citrate	310	284	10
	Citrate	310	294	100
	Citrate	306	256	10
	Citrate	306	261	100
	Tyrodex	294	266	100
	Saline	120	65	10
	Saline	115	55	10
	Saline	195	160	10
	Saline	100	80	10
	Saline	185	120	10
	Saline	140	90	10

Table III gives a summary of samples taken as soon as possible after mixture in silicone vessels. The time of sampling would vary from a few seconds to about three minutes depending upon the manipulations required in different experiments. In only three of the fifteen examples did the count of the heparin mixture correspond with that of the control mixture within the error of the method, and in two of these it was lower than the value for the control. This demonstrates the promptness of the heparin's effect.

TABLE IV EFFECT ON THE PLATELET COUNT OF MIXING HEPARIN AND BLOOD IN SILICONE VESSELS

HEPARIN AND HUMAN BLOOD IN SILICONE VESSELS						
EXPERIMENT A		EXPERIMENT B			EXPERIMENT C	
HEPARIN (10 U/C C)		HEPARIN (10 U/C C)		SALINE	HEPARIN (100 U/C C)	
TIME OF SAMPLE	PLATELETS × 1,000	TIME OF SAMPLE	PLATELETS × 1,000	PLATELETS × 1,000	TIME OF SAMPLE	PLATELETS × 1,000
Immed	125 (4%)*	Immed	140	220	Immed	120
3 min	105 (5%)	3 min	100	225	7 min	70
10 min	120 (18%)	7 min	60	165	15 min	75
16 min	80 (12%)	15 min	95	255	24 hr	50 (30%)
23 hr	110 (22%)	30 min	85	130	48 hr	70
		24 hr	70	Clotted		

HEPARIN AND CANINE BLOOD IN SILICONE AND PLAIN GLASS VESSELS						
TIME OF SAMPLE	SILICONE	SILICONE	SILICONE	SILICONE	SILICONE	PLAIN GLASS
	HEPARIN (0.1 U/C C)	HEPARIN (0.1 U/C C)	HEPARIN (10 U/C C)	HEPARIN (100 U/C C)	CITRATE CONTROL	HEPARIN (10 U/C C)
	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000	PLATELETS × 1,000
<i>Experiment D</i>						
Immed	220	319	218 (3%)	232 (2%)	312	276 (7%)
9½ min	192 (15%)	249 (21%)	204 (38%)	190 (16%)	325 (5%)	239 (51%)
30 min	225 (11%)	326 (51%)	150 (80%)	116 (76%)	184 (4%)	81 (69%)
2½ hr	7 (42%)	193 (39%)	97 (54%)	59 (84%)	231 (24%)	170 (66%)
24 hr	Clotted	62 (9%)	152 (30%)	99 (57%)	256 (12%)	122 (25%)
48 hr		Clotted	189 (19%)	122 (62%)	205 (5%)	143 (14%)
72 hr			210 (14%)	200 (41%)	221	137 (28%)
1 wk			108 (14%)	97 (29%)	304	150 (34%)
<i>Experiment E</i>						
Immed			284	294	310	259
10 min			191 (74%)	103 (85%)	210 (26%)	252 (61%)
30 min			129 (67%)	55 (80%)	178 (51%)	149 (73%)
3 hr			128 (51%)	93 (83%)	174 (46%)	133 (60%)
24 hr			27 (27%)	73 (27%)	200 (24%)	44 (23%)
48 hr			60 (12%)	104 (32%)	187 (24%)	101 (17%)
72 hr			41 (17%)	95 (17%)	193 (13%)	68 (21%)
<i>Experiment F</i>						
Immed			256 (77%)	261 (71%)	325 (3%)	295 (61%)
12 min			109 (93%)	250 (93%)	266 (20%)	101 (90%)
33 min			126 (85%)	109 (91%)	239 (25%)	215 (88%)
3 hr			159 (43%)	199 (86%)	295 (8%)	259 (50%)
24 hr			219 (40%)	195 (37%)	279 (4%)	180 (22%)
<i>Experiment G (Saline in Place of Citrate Control)</i>						
Immed			65 (4%)		120	
3 min			40		100	
7 min			45 (33%)		110	
15 min			120 (88%)		Clotted	
30 min			130 (77%)			
<i>Experiment H (Tyrode's Solution in Place of Citrate Control)</i>						
Immed				266	294	
6 min				229 (49%)	195	
23 min				108 (75%)		

\*Percentages in brackets are agglutination

In Experiments A B C and G platelet counts were made in the 1/100 dilution.  
In Experiments D E F and H platelet counts were made in the 1/10 dilution with urea.



Table IV gives the results obtained in later samples and attention is directed to the following points

(1) The decline initiated at mixing was continued to a low point depending on the time of sampling but varying from three minutes to twenty four hours

(2) In some instances a recovery occurred from this low point which could hardly be accounted for on the basis of irregular sampling

(3) Citrate controls observed for twenty four hours or more in three experiments showed a similar decline and recovery but less marked. Even a control in saline would sometimes show this within the half hour although the red and white cell counts were within the error of the method

(4) As in the experiments in vivo there appeared to be an individual variability

These findings raise several points of interest which will be discussed later

TABLE V PLATELET COUNTS AFTER HEPARIN IN VIVO AND WHEN HEPARINIZED BLOOD IS ADDED TO FRESH HEPARIN (10 UNITS C.C.) IN SILICONE VESSELS

TIME AFTER MIXING		SILICONE CONTROL	IN SILICONE VESSEL	AGGLUTINATION
<i>Experiment 16 2</i>				
-	Sample for silicone	115 000	Count in silicone	
3 min.			55 000	
10 min.			65 000	
15 min.			75 000	
30 min.			85 000	
	Heparin injected intravenously 1 000 units/kg		100 000	
-			Count in vivo	
3 min.			120 000	
10 min.			30 000	
30 min.			115 000	
60 min.			130 000	
-	Sample for silicone		Count in silicone	
-		135 000	135 000	
3 min.		130 000	135 000	5%
10 min.		125 000	90 000	
30 min.		135 000	95 000	
		100 000	115 000	30%
<i>Experiment 16 5</i>				
-	Sample for silicone	195 000	Count in silicone	
5 min.			160 000	
15 min.			80 000	
30 min.			55 000	
	Heparin injected intravenously 10 unit /c c of blood		120 000	
-			Count in vivo	
10 min.			220 000	
60 min.			50 000	
	Heparin injected intravenously		195 000	
-			185 000	10%
10 min.			60 000	25%
60 min.			150 000	13%
	Heparin injected intravenously 10 units/c c 15 min later		150 000	
10 min.			90 000	
60 min.			140 000	
-	Sample for silicone		Count in silicone	
-		150 000	150 000	
5 min.		120 000	90 000	
15 min.		150 000	80 000	28%
60 min.		170 000	85 000	

Platelet counts made in the 1/100 dilution

As a further test of the effect of heparin on platelets, a comparison was made of the counts *in vivo* with counts on the same blood *ex vivo*. Using the silicone technique, blood from a dog was added to heparin in a beaker and then heparin was injected into the same animal to bring the concentration in the circulating blood to approximately that in the beaker. An hour later another sample of blood was withdrawn and added to heparin in a silicone beaker. The results for two experiments are shown in Table V. Here it will be noted that

(1) The reduction that occurred immediately after mixing the first sample in the silicone vessel continued to a low point at seven minutes and reached a level approximating that found three minutes after injection into the animal.

(2) Blood drawn from the heparinized animal did not show the usual reduction in platelets immediately after mixing with fresh heparin in the beaker.

(3) In the second experiment three successive injections were each followed by a sharp decline in the platelets although at the time of the last two injections the blood was incoagulable.

(4) The counts in the second animal at the time of each injection were at successively lower levels and declined less with each successive injection.

Platelet counts were made by one of us (E. F.) in connection with experiments on heparin at Toronto in 1938-1941. On one occasion samples of blood which were taken into heparin instead of formalin-citrate gave unexpectedly low counts together with clumping. As a consequence, this technique was never repeated. On the other hand, there were experiments in which samples of blood which had been taken ten or fifteen minutes after injections of heparin showed no significant drop, although in the light of present experience some reduction would have been expected. These facts, together with some differences between our results and those of Wright,<sup>8</sup> to be discussed later, raised the question of differences in samples of heparin and the possibility of an impurity in the commercial product we were using. No evidence of the latter possibility was found in fractional precipitation with brucine. There was no concentration of the agglutinating activity in any one fraction. After discussing the earlier experiences with Dr. A. M. Fisher\* and Dr. A. F. Charles,\* they very kindly provided certain experimental lots for comparison with the commercial product. Also through the kind cooperation of Dr. Tage Astriup a sample of Heparin Leo was obtained in powder form and was compared with a similar product from Dr. Charles. The results of this study are contained in Table VI.

Heparin I, the commercial sample, was already in solution in 10 c.c. vials with 0.3 per cent tricresol as preservative. Samples II to VI were experimental lots, but II differed from III only by the absence of tricresol. Samples VII and VIII were freshly dissolved in 0.9 per cent saline with 0.3 per cent tricresol and were used within an hour after solution. Sample VII assayed 110 units per milligram and sample VIII, the Heparin Leo, 100 units per milligram. All samples were given intravenously to dogs under Nembutal in a dose of 100 units per kilogram, and platelet counts were made before injection and at approximately four, ten, thirty, and sixty minutes afterwards. The figures are adjusted to the same basis relative to the erythrocytes. The following features are apparent in Table VI.

\*Connaught Laboratories

- (1) One animal, C was distinctly more sensitive than the other three  
 (2) With one exception the minimum agglutination coincided with the lowest count  
 (3) With one exception the lowest count was obtained in the first sample after injection

TABLE VI PLATELET AND LEUCOCYTE COUNTS WITH DIFFERENT SAMPLES OF HEPARIN GIVEN INTRAVENOUSLY TO DOGS

EXPERIMENTAL GROUP	ANIMAL A			ANIMAL B			ANIMAL C			ANIMAL D		
	PLATELETS		W B C x 1000	PLATELETS		W B C x 1000	PLATELETS		W B C x 1000	PLATELETS		W B C x 1000
	x 1000	AGGLUTINATION (%)		x 1000	AGGLUTINATION (%)		x 1000	AGGLUTINATION (%)		x 1000	AGGLUTINATION (%)	
I	334	0	77	275	0	111	261	0	98	25	0	53
	22	67	15	45	77	33	22	80	17	20	55	17
	59	54	69	49	55	66(a)	21		56	62	63	36
	74	12	62	260	27	88	140		0	125	25	36
	348	0	54	273	2	90	68	12	61	49	5	53
II	216	5	95				236		116			
	191	23	108(b)				105	1	12			
	64	2	83				195	1	10(c)			
	68	0	103				206	0	128			
	210	0	80				222	2	124			
III	205	4	106				230	8	9			
	186	19	105				133	1	93			
	253	1	103				163	1	95			
	46	0	96				261	2	98			
	65	0	91				277	0	109			
IV	241	5	85				230	2	105			
	171	16	93				117	46	94			
	205	3	96				206	24	11(d)			
	244	2	71				248	0	14			
	49	3	61				229	0	62(e)			
V	187	8	74				208	6	109			
	195	15	95				81	54	108			
	158	3	67				137	21	115			
	201	2	63				258	0	123			
	183	8	61				236	0	118			
VI	194	1	71				234	0	106			
	18	13	79				126	23	125			
	241	0	87				186	1	110			
	31	0	63				299	0	139			
	240	0	45				314	0	131			
II							241	0	104	362	0	128
							47	29	114	190	49	113
							91	17	107	276	8	91
							287	1	132(f)	394	2	117
							314	0	122	362	0	94
II							221	0	89	349	0	124
							42	30	91	86	0	122
							11	18	91	260	14	108
							244	1	107	360	0	117
							300	0	112	390	1	84

Blood samples taken before injection and at four ten thirty and sixty minutes after except as follows (a) at eleven and one half minutes (b) at three and one half minutes (c) at eleven minutes (d) at sixteen and one half minutes (e) at sixty two and one half minutes (f) at thirty and one half minutes.

Blood diluted 1/5 in formalin citrate This 1:5 mixture diluted 1/10 with per cent urea solution for Heparin dosage 100 units per kilogram

(4) The sharpest reaction occurred with the commercial sample of heparin and was accompanied by a definite leucopenia

(5) Some experimental products given in the same dosage of anticoagulant units as the commercial one had much less effect upon platelets

#### DISCUSSION

These results agree with those of Copley and Robb<sup>2</sup> that the injection of commercial heparin into dogs will cause a transient thrombocytopenia. This result also has been found in man, as one of the authors (L. B. J.) demonstrated upon himself. Copley<sup>3</sup> has found platelet emboli in the rabbit and hamster following injection of heparin. Hence the phenomenon appears to be a general one, although in mice Copley and Robb<sup>2</sup> did not demonstrate the platelet change as clearly as in dogs. In the latter they found the maximum thrombocytopenia to be about 40 per cent, whereas in seven of our dogs the decrease reached 90 per cent or more. This may be due to the fact that their samples were not taken until twenty-six or more minutes after injection, although in three of their animals studied at shorter intervals the lowest platelet count occurred in the first twenty minutes.

The lowest counts in our experiments appeared within three and one half to five minutes after intravenous injection. A slow rate of injection, 1,000 units per kilogram per minute, was practically as effective as a rapid rate, 1,000 units per kilogram per second. In the human subject, subcutaneous administration appeared to delay and prolong the effect of heparin but not to abolish it. Continuous injection at 1 unit per kilogram per minute held the platelet count down, though not at the low point reached by a single injection. During the period when the blood was incoagulable from one injection of heparin, a second injection would again depress the count. In the first experiment, in Table V, the dose of 1,000 units per kilogram would prolong the clotting time to about eight hours, but the count in this animal returned to normal in one hour. Compared with the experiment shown in Fig. 1, the return of the count was more rapid in spite of the larger dose. This is another example of the individual variability encountered among dogs, but both instances favour the idea that some substance may render the heparin innocuous to platelets without inhibiting the anticoagulant power, or that the agglutinating action is a weaker property. It is interesting that the heparinized blood, drawn from the animal in Table V, when added to fresh heparin in the silicone beaker did not show the immediate drop in platelets commonly found with normal blood. This might mean greater resistance acquired by the platelet or perhaps the disappearance of sensitive platelets due to the injected heparin.

On the question whether the extent of the decline in vivo depends upon dosage, our data do not suggest any correlation. In Table I it will be seen that there was practically no difference in the extent of response to 50 units per kilogram in one animal and 1,500 units per kilogram in another, although in the latter instance there is evidence of a more prolonged effect if compared with the preceding experiment where 100 units per kilogram were used. In both Copley and Robb's<sup>2</sup> results and our own the differences point to individual variability.

In vitro however, where the same sample of blood can be used with varying doses Copley and Robb<sup>1</sup> showed that in strengths from 0.05 to 100 units per cubic centimeter the platelet count decreased as the heparin increased. Also they noted that this effect of heparin did not appear when it was added to citrated blood.

The mechanism of the swift disappearance and return of the platelets from the general circulation is not clear. The appearance of clumps of platelets in blood samples taken soon after the injection suggests that a filtering out of clumps in the capillaries is the cause of the sharp fall in vivo. But actual lysis may also be a possibility, and the increase in adhesiveness leading to agglutination may be merely an early stage of lysis. Wright<sup>8</sup> in her study of adhesiveness, noted only about a 10 per cent decline in the count in eighty minutes when using heparin with blood in paraffined tubes. It is admitted that our method of estimating agglutination is only a rough approximation but if the data of Experiments D, E, and F Table IV, are examined it will be noted that the maximum agglutination occurred in the ten or thirty minute samples. Where the heparin used was 10 units per cubic centimeter or more considering samples only up to three hours, the maximum agglutination either coincided with the lowest count or appeared in the sample immediately preceding it. All samples of twenty four hours or later showed a decline in agglutination from the maximum. This corresponded with a rising tendency in the counts in two of the blood specimens (Experiments D and F), though in Experiment E the counts tended to remain low. This inclines us to the opinion that clumps can break up again, especially in the living animal where there may be some factor tending to neutralize the heparin effect as suggested in Table V. On the other hand we have observed in late samples large clumps in which it was impossible to count individual platelets and which gave the suggestion of coalescence as though undergoing a slow form of lysis.

Lysis is also suggested in the mixtures in silicone because samples taken simultaneously from the center, bottom and side of the beaker gave counts which were all within experimental error. For example in one experiment the count from the center of the beaker was 100,000 from saline 80,000 after three minutes in heparin, and 45,000 after thirty minutes in heparin. Other samples taken at the same time from side and bottom were within plus minus 5,000 per cubic millimeter. If the decrease in the count were due to settling of clumps or their adhesion to the walls of the vessel one would expect a greater variation.

Against the idea of lysis of the platelets as a major factor in the decline are first, the very rapid recovery and, second the absence of any symptoms which might be expected from materials known to be present in platelets (thromboplastin vasoconstrictor substances, and so on).

The rapid return of the platelet count in some of the experiments in vivo was remarkable. In some cases after declining 60 to 75 per cent it reached the normal level again within an hour. This scarcely can be due to new formation of platelets. Lawrence and Valentine<sup>10</sup> estimated production in the cat to be about 1,600 to 2,800 per cubic millimeter per hour. Tocantins<sup>11</sup> found 40,000 per cubic millimeter per hour following splenectomy but this rate could hardly

be expected from normal bone marrow. A count rising from 20,000 to 228,000 in thirty-two minutes (Table I) does not look like new formation. It may be noted also that since the count before heparin was 295,000 no great destruction by lysis is indicated.

The fact that the leucocytes tend to decline when the platelet count is very low suggests that they also are caught with the platelet clumps in the capillaries. In one experiment, freshly drawn blood was mixed with heparin in the syringe, placed in a plain glass test tube, and kept fifteen minutes at body temperature before reinjection. After the blood had been drawn back into the syringe, it was found that the wall of the tube had a deposit of fine granules which, when washed off in formalin-citrate, proved to be platelet clumps. Many of these were adherent to leucocytes.

It is unlikely that there is any direct destructive action of heparin on the leucocytes since counts of the latter were made on all samples in Experiments D, E, and F (Table IV) and failed to show the parallelism with the platelet counts usually found in vivo.

The silicone experiments require further consideration. They were planned as controls of the in vivo experiments on the assumption that since contact with glass could be avoided, the conditions outside the body would resemble those within. The data did not support this assumption. The citrate controls in silicone listed in Table IV showed a 41 and 44 per cent reduction in two cases and 27 per cent in the third where the citrate was raised to 3.8 per cent of the final mixture. In another experiment not shown, an ovalate control in plain glass gave a 40 per cent reduction. Where heparin was the only anticoagulant present at 10 units or more per cubic centimeter, the platelet fall in silicone ranged from 56 to 75 per cent in Experiment D, 81 to 90 per cent in Experiment E, and 57 to 66 per cent in Experiment F, so that the decline in all three was definitely greater than with citrate or ovalate. A plain glass surface may be considered a uniform stimulus for platelet change, and in tests with heparin Wright found a reduction of 55 per cent to over 70 per cent compared with 10 per cent in the paraffin control. We found it rather surprising, therefore, that there was not sufficient difference between plain glass and silicone vessels to justify the thought that the character of the surface is an important factor in the reduction of platelets when anticoagulants are present. This result, together with other experiences with silicone, leads to the suggestion that at the moment of mixing certain changes affecting the platelet already may have been initiated and are continued even though at a retarded rate and to a limited extent after the addition of anticoagulants.

It should be pointed out that good blood samples for the silicone technique are more difficult to obtain from dogs than from human subjects. This is emphasized by the careful experiments of Patton, Ware, and Seegers.<sup>12</sup> Since Wright's tests were made with human blood, the differences mentioned between her results and ours may be related to this fact. Another variable which may be involved is suggested by the work of Binkhous<sup>13</sup> who has presented evidence of a plasma factor necessary for the lysis of platelets; it is not difficult to imagine agglutination as a related phenomenon. In experiments on dogs receiving

Diemariol but not recorded here, we have noted marked differences in the response of platelets from different animals to the same dose of heparin *in vitro*. It is evident, therefore, that these experiments with silicone cannot be regarded as simulating conditions within the blood vessel. Besides the changes in the blood incident to its removal, such factors as mixing and carbon dioxide tension are quite different *in vitro* compared with *in vivo*.

One of the puzzling features of the *ex vivo* experiments, illustrated in Table IV, was the return of the platelets after an initial decline when the blood mixtures were followed for twenty four hours or longer. This did not occur in all blood samples as for example, in Experiment C where the count remained consistently low from twenty four to seventy two hours. Even over shorter periods of time late samples might show increases over earlier ones as in Experiment G and occasionally even a saline control in silicone might show it as in Experiment B. Undoubtedly this was partly due to uneven distribution but the counts of red and white cells made at the same time did not show such wide variations. The decline in agglutination suggested one reason for an increase but the platelets in the aging blood could not always be recognized as old and indeed one had to consider again whether platelets can be formed from red cells.

In Schilling's "ideal" red cell a review of which is given by Ulpts<sup>14</sup> two structures are indicated which easily may be counted as platelets, and indeed one of them was so labelled by Schilling. Occasionally the ghost of a red cell surrounding such a structure will enable one to eliminate it from the count, but it is possible that the inclusion of some of these Schilling bodies may be partly responsible for the rise of the count in late samples. But there cannot be a great many of these "ideal" erythrocytes because platelet counts made by the use of 5 per cent urea solution which hemolyzes red cells showed general agreement with platelet counts made in the usual formalin citrate mixture being sometimes lower and sometimes higher. Since the use of urea allows a dilution of only  $\frac{1}{40}$  of the blood sample while the other count is made in the  $\frac{1}{400}$  dilution there was a much greater chance of the former showing an increase if many erythrocytes containing Schilling bodies were present. Rees and Ecker<sup>1</sup> called these refractile structures Arnold bodies, and the addition of formalin to the 3.8 per cent sodium citrate which they recommended made the laking of red cells less frequent. Irregularities in platelet counts made in the  $\frac{1}{400}$  dilution suggest that the red cells may interfere with a free distribution of platelets *when clumps are present* and the error is multiplied when compared with the lower dilution. For this reason reliance has been placed on the count made in the urea solution and the values given in Table VI were so obtained.

With regard to Table VI it should be pointed out that the barbiturate may have some slight influence on the figures. The effect of this type of anesthesia in lowering the erythrocyte count is well known. During 1941-1942 in some experiments that were being made at Toronto by Dr. R. E. Haist on surgical shock, one of us (D. F.) noted the usual reduction in red cells following the barbiturate but found the platelets were not reduced in the same proportion. This has the effect of a relative increase and is quite definite when compared with preanesthetic counts. There was an indication that a slight relative increase might continue for

a time in successive samples after anesthesia. This may be the reason why in Table VI some of the experiments show at thirty or sixty minutes a higher count than the initial one. We mention this as a caution in case this increase be interpreted as increased production in immediate response to the heparin. The anesthesia avoids the excitement incident to the taking of each sample in untrained dogs, and since the important count is within five minutes after heparin and is usually only a few minutes after the initial count, conditions appear more favorable for a comparison between different samples of heparin.

Platelets are sensitive to many foreign materials and it should be recalled that the synthetic anticoagulants studied by Astrup and Piper<sup>16</sup> also caused platelet agglutination, but the fact that heparin brings about this phenomenon has the appearance of a physiologic contradiction. The formation of platelet emboli does not seem to harmonize with the prevention of platelet thrombi. However, the very act of purification may cause commercial heparin to differ from that released within the animal body. The results shown in Table VI suggest that more work is required before platelet agglutination can be accepted as a property of natural heparin.

The effect of commercial heparin on platelets resembles its effect on chylomicrons seen in studies of lipemia by Waldron and Friedman.<sup>17</sup> It is possible that there is some common underlying factor for both these actions.

#### SUMMARY

Heparin on intravenous injection in man or dog may cause a brief thrombocytopenia. In dogs this may reach 10 per cent of the original count, although there is individual variability. The fall in the platelet count is repeated with repeated injections. The decline is also observed when heparin is added to blood in silicone vessels. Some experimental lots of heparin in the same dosage of anticoagulant units have shown much less effect upon platelets than the present commercial product.

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## THE EFFECT OF HEPARIN ON PLATELETS IN VIVO

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WHEN either peptone or anaphylactic shock is produced in dogs, the platelets are temporarily but precipitously diminished, heparin is liberated into the blood, and histamine or a histamine-like substance is produced.<sup>1</sup> This triad has become of particular interest since Allen and Jacobson<sup>2</sup> have found that dogs exposed to highly ionized radiation suffer a marked thrombocytopenia, heparinemia, and a peripheral capillary dysfunction manifested by petechiae. In a recent note Copley<sup>3</sup> implies that the heparin is responsible for the thrombocytopenia and that it "may also take part in the formation of the petechial hemorrhages." He cites his work and that of his associates in which it was shown that heparin causes an agglutination *in vitro*<sup>4</sup> and likewise an agglutination and subsequent thrombocytopenia *in vivo*.<sup>5</sup> As the result of these observations it becomes important to determine whether heparinemia produced either endogenously or exogenously causes thrombocytopenia, or whether the fall in platelets and the outpouring of heparin in shock and after high ionizing irradiation are concomitant occurrences resulting perhaps from a common agent. As a preliminary approach to this problem, the effect of intravenously administered heparin on the number of circulating platelets was studied.<sup>§</sup>

### EXPERIMENTAL

The action of heparin on the platelet count was determined in rabbits, dogs, and men. The heparin employed was obtained from several pharmaceutical companies in the form of 10 c.c. vials each containing 100 mg. of the sodium salt of heparin. Several of these preparations have their potency stated in terms of Toronto units (1 mg. of the sodium salt of heparin is equivalent to 110 units).||

The heparin was injected intravenously at a constant slow rate of approximately 1 to 2 c.c. per minute. In man only one standard dose was studied, namely 0.8 mg. (88 Toronto units) per kilogram of body weight, since this approximates the one generally employed for prophylaxis against thrombosis. In dogs and rabbits the doses ranged from 0.5 to 5 mg. (55 to 550 units) per kilogram of body weight. Samples of blood were taken before and at fixed intervals after the heparin was given. The vein into which the heparin was injected was not

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§After this investigation was completed we were informed by Dr. Charles H. Best that Dr. Edward Fidler and Dr. Louis Jaques had made a similar study. Dr. Fidler kindly supplied us with a summary of their findings which are similar to ours. They too observed the rapid agglutination of platelets in dogs following the injection of heparin. See p. 1410.

||We are indebted to Dr. K. K. Chen of Eli Lilly & Company, Indianapolis, Ind.; Dr. John T. Correll of The Upjohn Company, Kalamazoo, Mich.; Dr. George R. Hazel of the Abbott Laboratories, North Chicago, Ill.; Dr. Merton Lockhart of Lederle Laboratories, Inc., New York, N. Y.; and Dr. Kenneth W. Thompson of Roche-Organon, Inc., Nutley, N. J., who kindly supplied us with vials of heparin from their respective companies.

used for subsequent collection of blood. A silicone coated syringe was used and the blood for the platelet count was transferred immediately to a silicone coated test tube immersed in ice water. An alternative method consisted of placing 0.2 cc of 3.8 per cent sodium citrate directly in a 1 cc syringe and drawing blood exactly to the mark. The platelets were determined by direct count. The blood was diluted 1:200 with 3.8 per cent sodium citrate containing 0.2 cc formaldehyde (40 per cent) per 100 cubic centimeters.

In man the change in heparin concentration can be successfully followed by the coagulation time (Lee White), the prothrombin time and the thrombin titration.<sup>6</sup> In rabbits and dogs only the last method is satisfactory since the prothrombin time is too little affected and the coagulation time is so markedly prolonged that it becomes impractical to perform and it is difficult to achieve accuracy.

### RESULTS

The findings obtained by injecting heparin into man are given in Table I. It will be observed that the platelet count remained unaltered and that all of the heparin was removed from the blood in three to four hours. The bleeding time remained normal even during the time the heparin concentration in the blood was highest.

TABLE I. THE EFFECT OF INJECTING HEPARIN INTRAVENOUSLY IN MAN ON THE PLATELET COUNT, COAGULATION TIME, PROTHROMBIN TIME AND THROMBIN TITRATION

TIME	SUBJECT J N S (WEIGHT 80 KG) HEPARIN 64 MG *				SUBJECT M S (WEIGHT 63 KG) HEPARIN 50 MG *			
	PLATE LETS (THOU SANDS)	COAGU LATION TIME (LEE WHITE) (MIN)	PRO THROM BIN TIME (SEC)	THROM BIN TITRA TION (1 S) †	PLATE LETS (THOU SANDS)	COAGU LATION TIME (LEE WHITE) (MIN)	PRO THROM BIN TIME (SEC)	THROM BIN TITRA TION (1/5 S) †
0	186	5 3/4	12	7	116	6	12	65
5 min.	168	60	27	250	122	42	22	125
15 min.	180	40	19	210	120	32	17.5	65
0 min.	173	35	17.5	50	113	30	16.0	32
1 hr	178.5	20	15.5	49	118	20	14	20
2 hr	169	13	13.5	36	115	11	13	8
3 hr	161	12	14	12	110	7	12.5	6
4 hr	164	7 1/2	12	7 1/4				

Hoffman La Roche. Each subject received 0.8 mg of heparin per kilogram of body weight.

†The thrombin was prepared according to the directions of Eagle and modified by Quick.<sup>7</sup> The product obtained which is designated as full strength (F S) will clot two parts of oxalated human plasma in three seconds. When the thrombin is diluted 1:5 (1/5 S) it clots plasma in seven seconds.

In Table II the effect of intravenously injected heparin on dogs is recorded. It will be observed that the five brands of heparin had approximately the same effect in reducing the number of circulating platelets. A dose of 0.5 mg (55 units) per kilogram of body weight seems to be the critical amount since at some times produced thrombocytopenia and sometimes did not. Above this dose, a fall in platelets unfailingly was observed.

To determine how soon the platelets are affected after the injection of heparin blood was taken every minute for five minutes. One minute after the injection all the platelets were clumped but still in the blood stream. A minute later single platelets again appeared and the clumps were diminishing. At the end of five minutes all agglutinated platelets had disappeared from the blood (Table III).

TABLE II THE EFFECT OF INJECTING HEPARIN INTRAVENOUSLY IN DOGS ON THE CIRCULATING PLATELETS

BRAND OF HEPARIN	ABBOTT	ABBOTT	HOFFMANN LA ROCHE	EDERLE	IIIY	JOHN	HOFFMANN LA ROCHE			
DOSE PER KG OF BODY WEIGHT	2 MG	1 MG	1 MG	1 MG	1 MG	1 MG	0.5 MG			
DOG	F	S	S	M	G	M	F			
	THROM BIN TITRA TION (1/2 S) (SEC)	THROM BIN TITRA TION (1/2 S) (SEC)	THROM BIN TITRA TION (1/2 S) (SEC)	THROM BIN TITRA TION (1/2 S) (SEC)	THROM BIN TITRA TION (1/2 S) (SEC)	THROM BIN TITRA TION (1/2 S) (SEC)	THROM BIN TITRA TION (1/2 S) (SEC)			
TIME (MIN)	0	391	397	425	262	219	341	352	6	
5	25	33	20	132	23	75*	111	29	172	
15	247	27	11	300	105	111	341	21	94	
30	295	21	8.5	391	13	154	354	14.5	371	9

\*Moderate agglutination

TABLE III THE SPEED WITH WHICH PLATELET AGGLUTINATION AND THROMBOCYTOPENIA OCCUR AFTER INJECTION OF HEPARIN IN THE DOG (DOG G, WEIGHT 16 Kg HEPARIN INJECTED 16 MG)

TIME (MIN)	PLATELETS (THOUSANDS)	AGGLUTINATION
0	193	None
1		Complete
2	65	Nearly complete
3	25	Extensive
4	19	Little
5	25	None

\*Hoffman La Roche

The various brands of heparin produced no thrombocytopenia in rabbits as seen in Table IV. The platelet counts were made on arterial blood.

Only a few studies of the effect of injecting peptone in rabbits were made since they yielded results similar to the ones obtained either by Quick<sup>1</sup> or Baronofsky. Peptone causes a marked drop in the platelet count but produces little or no heparinemia (Table V) whereas in dogs enough heparin is often poured into the blood to render it incoagulable.

From the results obtained in the present study one can conclude that the intravenous injection of heparin in doses as large as 5 mg. per kilogram of body weight in rabbits and 0.8 mg. in man causes no demonstrable diminution of the circulating platelets. In dogs, on the contrary, doses as small as 0.5 mg. per kilogram of body weight may cause a marked but transient fall in the platelet count. This is due to the agglutination of these cells and presumably to the subsequent removal of these clumps by the capillary bed. The agglutination as shown in Table III occurs almost immediately after the heparin is injected. Promptly following this reaction restoration of the platelet count begins to occur and may be amazingly rapid. In some instances the count may be one third of the original level five minutes after the injection and completely normal ten minutes later.

Since heparin does not cause thrombocytopenia in either man or rabbit it is clear that this agent per se has no influence on platelets. To imply that the thrombocytopenia following high ionizing irradiation is the resultant of heparinemia is certainly open to question. To be sure the injection of peptone in dogs causes heparinemia, thrombocytopenia, and histamine production but in the rabbit the same dose of peptone produces only thrombocytopenia. It is unlikely that the heparinemia in dogs is the cause of the abrupt drop of platelets. It might also be stated that injection of histamine likewise causes no change in the platelet count<sup>7,8</sup> consequently this agent can not be implicated in the thrombocytopenia observed in shock. On the basis of present evidence it seems reasonable to conclude that the three effects of shock seen in dogs are independent of each other except that their causative agent may perhaps be the same. One must not ignore the possibility however that the coagulation defect which we have recently demonstrated in thrombocytopenia<sup>9</sup> when accentuated by heparinemia may convert the vascular hyperpermeability due to histamine into a serious hemorrhagic condition. It is doubtful if heparin alone or even in the sole presence of a thrombocytopenia will cause petechiae or a prolonged bleeding time. These later effects assuredly are the result of a vascular factor.

TABLE IV THE EFFECT OF HEPARIN GIVEN INTRAVENOUSLY ON THE PLATELET COUNT IN THE RABBIT

BRAND OF HEPARIN	ABBOTT		ABBOTT		HOFMANN LA ROCHE		LEDERLE		LILLA		UPJOHN	
	5 MG		2 MG		2 MG		2 MG		2 MG		2 MG	
DOSE PER KG OF BODY WEIGHT												
TIME (MIN)	THROMBIN TITRATION ( $\frac{1}{2}$ S) (SEC)		THROMBIN TITRATION ( $\frac{1}{2}$ S) (SEC)		THROMBIN TITRATION ( $\frac{1}{2}$ S) (SEC)		THROMBIN TITRATION ( $\frac{1}{2}$ S) (SEC)		THROMBIN TITRATION ( $\frac{1}{2}$ S) (SEC)		THROMBIN TITRATION ( $\frac{1}{2}$ S) (SEC)	
	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)	PLATELETS (THOU SANDS)
0	500	4	320	4	268	3½	189	4	271	4	188	4
5	478*	30	290	12.5	259	24	187	26	289	22	177	24
15	496	22	316	9.5	247	13	210	18	398	12	181	13
30	425	13	390	5.5	275	59½	164	10.5	270	8.5	201	9.5

\*Slight agglutination

TABLE V THE INFLUENCE OF INTRAVENOUSLY INJECTED PEPTONE ON THE PLATELETS IN THE RABBIT (300 MG OF PEPTONE PER KILOGRAM OF BODY WEIGHT)

TIME (MIN)	PLATELETS (THOUSANDS)	AGGLUTINATION	THROMBIN TITRATION (1/8) (SEC)
0	219	None	65
5	125	Almost complete	65
15	60	Extensive	65
30	125	Moderate	65

Witte Rostock Germany

It is curious that heparin causes a thrombocytopenia in dogs but not in man or rabbits. One can proffer several possible explanations. The first is that the purified heparin may still contain a trace of impurity to which the dog but not the rabbit or man is susceptible. A second hypothesis is that heparin reacts with a plasma constituent, thereby producing an agent which causes the agglutination of platelets. According to this view dog plasma contains this factor but rabbit and human plasma do not. A third hypothesis is that dog plasma lacks protective factors that would counteract the physicochemical alteration induced by heparin. It is to be noted that the effect of heparin on the platelets is immediate and that the platelets actually begin to increase while the heparinemia is still at its height. This shows that the presence of even a high concentration of heparin does not depress the platelet count but actually allows a rapid restoration which is brought about very likely from the liberation of the individual cells from the agglutinated clumps.

There are probably two types of platelet agglutination. The first is the type induced by heparin in dogs by peptone and perhaps by many other agents as Achard and Aynaud noted as early as 1908<sup>10</sup>. Perhaps this type of agglutination is brought about by as simple a change as the removal of an electric charge needed to maintain the discreteness of the platelet. The second type of agglutination is apparently the result of labilization of the platelet by thrombin. In this type the platelets become sticky, readily adhere to rough surfaces, and disintegrate thereby liberating an enzyme that activates the plasma thromboplastinogen. The platelets remain stable whenever the production of thrombin is inhibited. This accounts for the stability of platelets in hemophilia, in mailed Dicumarol hypoprothrombinemia, in oxalated and citrated plasma and in heparinized plasma. Heparin however does not prevent the first type of agglutination as Quick, Ota and Baronofsky<sup>7</sup> and Fridlar and Waters<sup>11</sup> have shown.

From a practical point of view the agglutination of platelets by heparin is of little moment since it does not occur in man and since even in dogs it is very transitory and unaccompanied by any untoward effects. Heparin depresses platelet agglutination of the second type by inhibiting the formation of thrombin as well as by neutralizing it. Because of this action, heparin is a reliable drug for prophylaxis against intravascular clotting.

## SUMMARY

The effect of intravenously administered heparin on the circulating platelets was studied in man, rabbits, and dogs. Five brands of commercial heparin were used. No significant agglutination or thrombocytopenia was observed in man.

and rabbits even after relatively large doses. In dogs a dose of 1 mg per kilogram of body weight invariably caused agglutination of platelets and a subsequent transitory thrombocytopenia. Probable causes for this are discussed.

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## EFFECT OF VITAMIN K ON DICUMAROL INDUCED HYPOPROTHROMBINEMIA IN RATS

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MOST of the reports dealing with the effect of vitamin K on dicumarol induced hypoprothrombinemia have been based on one or another of the one stage methods of prothrombin estimation<sup>1</sup>. It has been shown that these methods depend not only on the concentration of prothrombin but also on the rate of conversion of prothrombin to thrombin<sup>2</sup>. Thus the one stage tests lose some of their reliability as a means of measuring prothrombin concentration unless factors which may influence conversion rate are eliminated. In recent years several workers have established that another constituent present in normal plasma influences the conversion of prothrombin to thrombin<sup>3,13</sup>. In addition the presence of a distinct inhibiting substance in the blood of animals treated with a hydrogenated derivative of Dicumarol has been postulated<sup>14,1</sup>. This substance is considered to be similar to but not identical with heparin. Some of the clinical reports are based on the cessation of spontaneous bleeding which is admittedly influenced by many variables.

The two stage method of prothrombin determination<sup>15</sup> separates the conversion phase from the clotting phase and thereby eliminates the rate of conversion of prothrombin to thrombin as a factor in prothrombin measurement. It seems pertinent, then, to examine by this method the effect of Dicumarol on the prothrombin level of blood and the modification of that effect by vitamin K.

### MATERIALS AND METHODS

Adult albino rats of the Sprague Dawley strain were used. They were maintained on a diet which contained adequate amounts of vitamin K. Water and diet were fed ad libitum.

Suspensions of 3,3 methylenebis (4 hydroxycoumarin) were prepared as follows: 70.2 mg Dicumarol† were suspended in 32.1 cc of distilled water. To effect increased dispersion of the drug suspension 3.0 cc of 0.1N sodium hydroxide were added.

Menadione‡ (2 methyl 1,4 naphthoquinone) was dissolved in corn oil (Mazola) so that 50 mg were contained in each cubic centimeter of the solution.

Hykinone§ (menadione bisulfite) was used as supplied by the manufacturer. One cubic centimeter contains the equivalent of 2.5 mg. of menadione.

Dicumarol and menadione were given by stomach tube and Hykinone was given by intraperitoneal injection. Both the vitamin K preparations and Dicumarol were given as daily doses.

Blood obtained by jugular puncture was drawn into 1.8% per cent potassium oxalate. The blood and anticoagulant were carefully mixed and placed in a hematocrit tube and the tube was set in ice water to induce rapid cooling. After centrifugation in a refrigerated room (5°C) prothrombin titration by the two stage method<sup>15</sup> was performed on the plasma.

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\*Rockland Rat Diet (Complete.)

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‡Eli Lilly & Company, Indianapolis, Ind.

§Abbott Laboratories, North Chicago, Ill.

## RESULTS

I *Dicumarol Alone*—A In the first experiment the animals were given daily doses of constant size of Dicumarol, and the prothrombin level (expressed in per cent of normal) was followed for a period of from two weeks in some

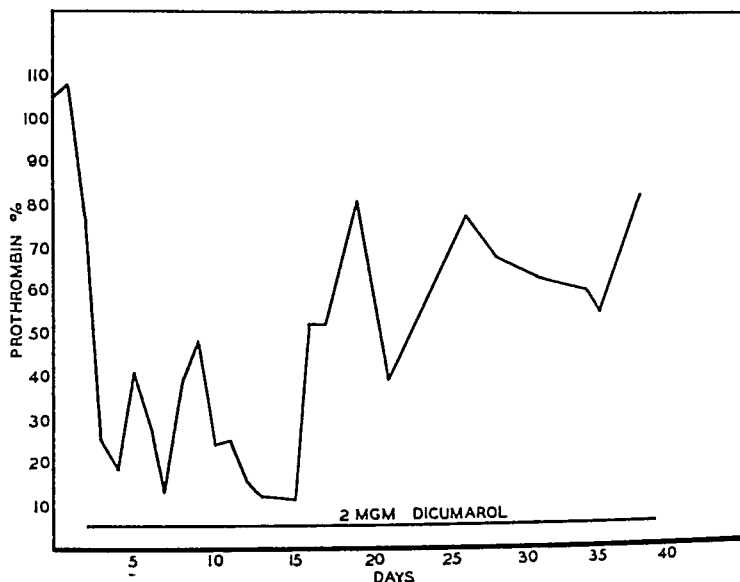


Fig 1 —The effect of daily doses of Dicumarol on the prothrombin level of rat plasma

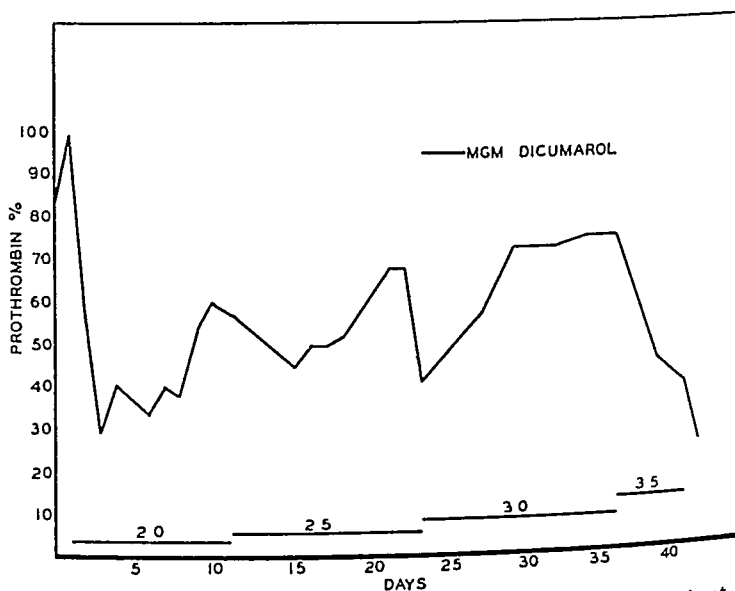


Fig 2 —The effect of daily doses of increasing size on prothrombin level of rat plasma.

cases to as long as forty-three days in others. Fig 1 shows a typical curve. There was a definite tendency for the animals to develop a tolerance to the drug. After approximately two weeks the prothrombin level tended to rise, and at the end of three to four weeks it commonly approached normal values. The daily variations in prothrombin level of Dicumarol-treated rats were great, as much

as 40 per cent in some instances. These variations are greater than can be explained on the basis of technical error in performing the determinations. Repeated determinations on many occasions showed that the variations in prothrombin level were genuine.

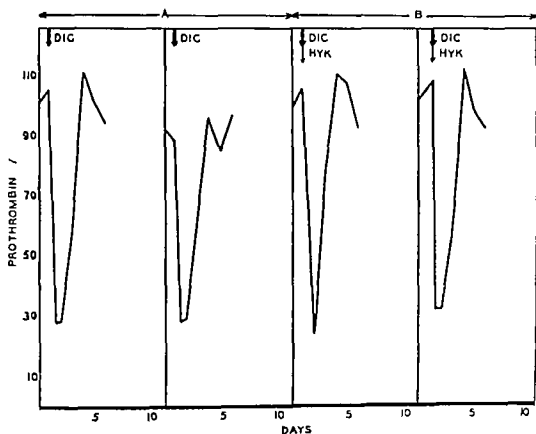


Fig 3—A Single dose of 4 mg Dicumarol  
B Single dose of 4 mg Dicumarol and 4 cc Hykinone

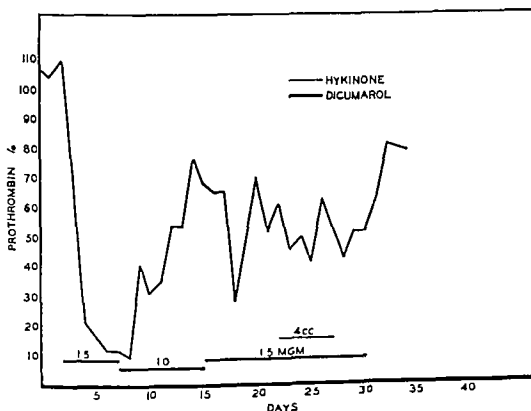


Fig 4—The effect of Hykinone given during administration of Dicumarol

B In another group of rats the dosage was gradually increased in an effort to keep the prothrombin at a low level. Virtually the same type of curve was obtained (Fig 2) although the prothrombin level tended to remain low for a longer time. Succeeding large doses had less effect than earlier small doses.

C In the third experiment the effect of a single dose of Dicumarol was studied (Fig 3, A) A dose of 40 mg of Dicumarol was given, and the prothrombin level was followed for two days after it had returned to normal The maximum hypoprothrombinemic effect was obtained within forty eight hours and recovery was complete within ninety-six hours after the time of medication

In the foregoing experiments the prothrombin level promptly returned to normal when Dicumarol administration was stopped This occurred within ninety-six hours after administration of the last dose of Dicumarol Recovery after repeated doses was at essentially the same rate as after a single dose

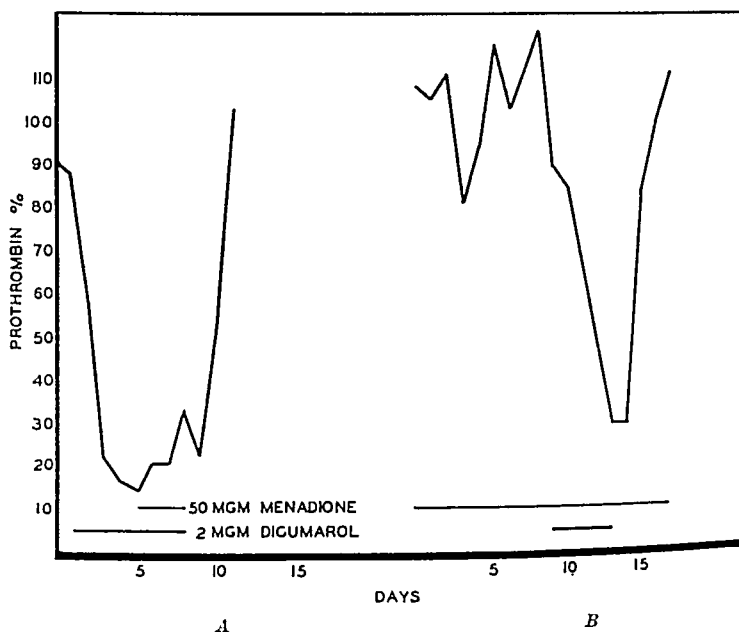


Fig 5—A Left-hand curve The effect of menadione given immediately preceding discontinuance of Dicumarol on the rate of recovery from the Dicumarol effect.  
B, Right-hand curve Menadione preceding during and after Dicumarol administration

II *Dicumarol and Vitamin K*—A Hykinone (40 cc) had no detectable effect when given to rats receiving daily doses of Dicumarol (Fig 4) No change was observed which was greater or in a different direction than frequently was observed with continued administration of Dicumarol alone

B In a second group of animals large doses of menadione (50 mg daily) were given for a period of one to two weeks before Dicumarol administration was begun, and it was continued during the period of Dicumarol administration (Fig 5, B) A standard dose of 20 mg of Dicumarol was given The hypoprothrombinemic effect of the Dicumarol in these animals was similar to that in animals which had received no menadione (Fig 1) Upon discontinuing the Dicumarol, recovery occurred at the same rate as in rats which had received no menadione

C A third group of animals was given Dicumarol for at least five and as long as twenty-four days During the last three to six days of Dicumarol administration 50 mg of menadione were also given (Fig 5, A) When both

Dicumarol and menadione were stopped the prothrombin level rose to normal at the same rate as it did when the administration of Dicumarol was stopped in animals which had received no menadione (Fig 6) In other animals in the

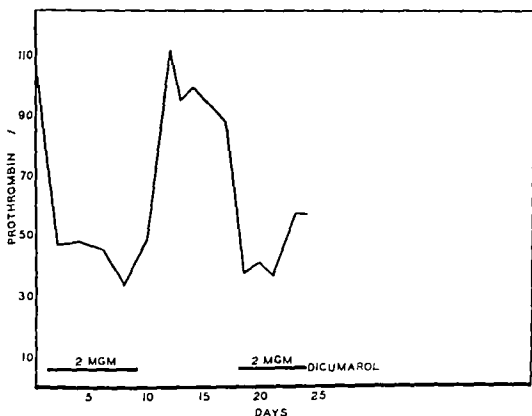


Fig 6—Rate of recovery from Dicumarol effect after drug was stopped

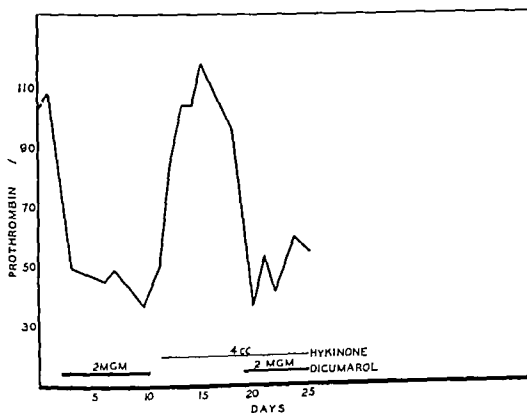


Fig 7—Rate of recovery from Dicumarol effect when Hykinone was given after the Dicumarol was stopped. Note that the prothrombin was reduced when Dicumarol was given again in spite of the continued administration of Hykinone.

same experiment vitamin K was given as Hykinone (dose equivalent to 10 mg menadione) intraperitoneally. The results were the same as with menadione in corn oil by stomach tube.

D In a fourth group of animals, Dicumarol was given for a period of five to ten days and then followed by the administration of 40 c.c. of Hykinone daily during the period of recovery (Fig 7). Recovery occurred at the same rate and to the same degree as in rats which received no Hykinone (Fig 6). When Dicumarol was administered immediately following complete recovery a response similar to that obtained with the initial dose was observed in both groups.

E In single dose experiments the prothrombin curves of rats given Dicumarol and Hykinone together (Fig 3, B) were similar to those of rats which received Dicumarol alone (Fig 3, A).

#### DISCUSSION

There are two outstanding differences in technique between these experiments and those reported by Overman and co-workers.<sup>2</sup> Their animals were given a diet low in vitamin K while ours were given a diet which contained adequate amounts of it. The prothrombin determinations in their experiments were done by a one stage method using dilute plasma while ours were done by the two-stage method. We wished to parallel as closely as possible clinical conditions in which the diet is not likely to be deficient in vitamin K, and to measure prothrombin concentration only.

The results of clinical studies of the effect of vitamin K on Dicumarol induced hypoprothrombinemia are confused somewhat because additional measures such as discontinuance of Dicumarol administration, blood transfusions, and other agents have been used to correct the hemorrhagic tendency. The clinical studies also lack the control possible in experimental animals. In many cases vitamin K was given at a time when the prothrombin level might have been expected to be rising due to the development of tolerance to the drug. Another possible cause for the great variation in clinical results is the wide diversity of methods used to estimate prothrombin. These vary from simple observation of cessation of spontaneous bleeding to the dilute plasma technique of Link. There is some evidence<sup>17</sup> that the Quick and Link methods cannot be substituted for each other. This suggests that they may measure different things. They, as all the one-stage methods, are certain to be influenced by plasma factors other than prothrombin concentration. There is also evidence<sup>18</sup> that the dilute plasma techniques have much greater standard deviations on duplicate determination than do whole plasma techniques. Even though the sensitivity is increased by dilution of the plasma, the increased possibility for error may be another reason for inconsistencies of results.

It is possible that vitamin K administration affects factors which govern prothrombin conversion as well as those which control its concentration. This could explain the protective action of vitamin K against Dicumarol which has been reported by workers using the one-stage methods of prothrombin estimation. It would also explain why this action is not demonstrable by the two stage method.

#### SUMMARY

1 The prothrombin level, as determined by the two stage method, varied considerably from day to day in rats treated with Dicumarol. There was a

definite recovery phase soon after the initial fall in prothrombin caused by Dicumarol. Later the animals showed a decided tendency to escape from the effect of the drug.

2 Menadione and menadione bisulfite in large doses had no detectable counteracting effect on the prothrombin level of rats whether given before, during, or after Dicumarol administration.

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# VENTRICULAR IRREGULARITIES INDUCED BY SYMPATHO ADRENAL DISCHARGE AND CHLOROFORM

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CHENOWETH,<sup>1</sup> working with dogs, reported that the inhalation of the vapor of various lipotropic hydrocarbons including toluene, xylene, petroleum ether, and gasoline, sensitizes the mammalian ventricle to epinephrine with resultant extrasystoles, tachycardia and fibrillation, simulating the well known chloroform-epinephrine syndrome.<sup>2 3 4 5</sup>

In discussing his findings, Chenoweth suggests that human subjects sensitized by the inhalation of such vapors might be susceptible to ventricular fibrillation, if subjected to emotional stress which would liberate epinephrine.

The electrocardiographic studies presented here, on dogs in which a sympatho-adrenal discharge was pharmacologically induced and to which chloroform was administered during the height of such induction, seem to substantiate that suggestion.

Dogs weighing 2 to 15 kilograms were put under basal anesthesia by intraperitoneal injections of sodium pentobarbital solution carrying 375 mg per kilogram of body weight. Control presurgical electrocardiographic records were then taken using the three standard leads. Tracheal and carotid cannulae were then inserted and the femoral vein was exposed, and after a thirty minute rest period following the surgery, an electrocardiographic Lead II was recorded. Each animal was given from 0.5 to 2.0 mg of atropine sulfate intravenously, depending on the weight of the animal, and five minutes later 15 mg of physostigmine salicylate. Control records (electrocardiographic Lead II) were taken three minutes after each drug injection. Five minutes after the physostigmine was given, 0.2 to 4.0 mg of acetylcholine bromide (depending on the weight of the animal) were injected. At the onset of the resulting blood pressure rise, the administration of chloroform vapor was begun and continued uninterruptedly until a danger point as indicated by marked fall in blood pressure and apnoea was reached. Continuous electrocardiographic Lead II, blood pressure and respiration records were obtained from the time just prior to the acetylcholine administration until the cessation of the chloroform anesthesia.

In this sequence of injections, presumably the muscarinic action of the acetylcholine would be blocked by the atropine and the destructive action of cholinesterase on the acetylcholine held in check by the physostigmine, leaving the nicotinic action of the acetylcholine unrestrained. The nicotinic effects of acetylcholine are believed to include a sympatho-adrenal discharge and the mobilization of epinephrine or an epinephrine-like substance.<sup>6, 7, 8</sup> If the nicotinic

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effect were great enough, it seemed that the intrinsic epinephrine liberated by the animal could affect the chloroform sensitized ventricular complex just as intravenous injections of epinephrine

Nine dogs of both sexes and various weights were studied. No differences in results attributable to sex or weight were found. There was however a wide variation in sensitivity as indicated by the fact that in three dogs a nicotine effect could not be induced, in spite of different dosages of drug administration.

In the six dogs in which nicotine responses were evoked the following abnormal electrocardiographic findings were obtained during the reaction to chloroform

- Three dogs Ventricular extrasystoles varying from single extra beats to short bursts of twenty or more ventricular extrasystoles
- Two dogs A persistent ventricular tachycardia of several minutes duration
- One dog A ventricular tachycardia changing to fibrillation during which the animal expired

In all instances except the last the normal electrocardiographic pattern returned following the termination of the abnormal cardiac activity.

Aside from the changes listed no other deviations in the electrocardiogram were noted and these changes occurred only during chloroform inhalation following the injections of atropine, physostigmine and acetylcholine. No such changes in electrocardiogram were noted following the injections of atropine, physostigmine, and acetylcholine into animals receiving no chloroform.

This was to be expected, for Myerson and co-workers<sup>9</sup> have shown that although physostigmine and choline ester may produce severe heart block atropine prevents this block. In the present experiments atropine and physostigmine always preceded the injection of the acetylcholine.

The findings reported here strengthen the suggestion of Chenoweth<sup>1</sup> that intrinsic epinephrine if liberated in sufficient quantity through emotional stress could present a serious hazard through its effect on the ventricular complex sensitized to various lipotropic hydrocarbons.

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## LABORATORY METHODS

### A MICROMETHOD FOR THE DETERMINATION OF THE HUMAN ALBUMIN, GLOBULIN, AND HEMOGLOBIN CONTENTS

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IN OUR previous communications<sup>1, 2</sup> it was reported that the estimation of human albumin in serum or other body fluids by means of the precipitin reaction gives an accuracy which approximates that of electrophoretic analysis, the generally accepted standard, and requires only a minute amount of the protein. This latter situation makes it possible for the development of a micromethod for the determination of albumin, total protein, and hemoglobin in capillary blood taken by finger puncture. These determinations were performed with 0.2 ml of blood which was then diluted with a known volume of 0.85 per cent saline solution, containing enough heparin to prevent coagulation. The red cells were then removed by centrifugation and laked with a dilute ammonia solution for hemoglobin determination with a photoelectric colorimeter. Aliquot samples of the supernatant were used for determination of total protein nitrogen and albumin nitrogen. The former was estimated by measuring the turbidity of the trichloroacetic acid precipitate, the latter, by the precipitin method. The difference between the total protein nitrogen and albumin nitrogen was taken as the total globulin nitrogen.

#### *Reagents —*

1. A dilute ammonia solution. Approximately 5 ml of concentrated ammonia solution (28 per cent ammonia by weight) are diluted with water and made to 1 liter.
2. A 7.5 per cent trichloroacetic acid solution. Seventy-five grams of Merck's reagent grade trichloroacetic acid are dissolved with water and made to 1 liter.
3. A normal saline solution, 17.0 grams of chemically pure NaCl are dissolved with water and then made to a volume of 2 liters. To one liter of this solution are added 10 mg of heparin.
4. Antihuman albumin rabbit serum. Sera of rabbits immunized against human albumin can be prepared according to our previously described procedure.<sup>1</sup> The pooled sera is kept in a frozen state until shortly before use, and filtered if necessary, after thawing. It is then diluted exactly with an equal volume of the saline solution.

#### *Procedure —*

Blood is drawn from the tip of the finger by puncturing with a dry lancet until it flows freely. A 0.20 ml sample is measured with a capillary pipette and transferred quantitatively into a 15 ml centrifuge tube containing 10.0 ml of 0.85 per cent saline solution with heparin. The suspension is then centrifuged for fifteen minutes, and the supernatant (referred to as SU) is carefully removed by a capillary tube and saved for the determination of the albumin nitrogen, and the total plasma protein nitrogen.

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*Determination of Hemoglobin —*

The red cells at the bottom of the tube are hemolyzed with 5 ml of the dilute ammonia solution. After taking 4 ml of the hemoglobin solution are then pipetted into a Klett Summerson tube containing 4 ml of the same ammonia water. Thus the final sample contains an equivalent of 0.0133 ml of blood. The amount of hemoglobin is then determined colorimetrically, using a green filter of 540 millimicrons. To express the results in grams per cent (see columns A and B in Table I) it is necessary to determine the oxyhemoglobin factor by correlating the colorimeter readings with oxygen combining capacity.

*Determination of Total Protein Nitrogen —*

Duplicate samples of 3.0 ml of the supernatant (SU) are pipetted into two Klett Summerson tubes to each of which are then added 2.0 ml of 1 per cent trichloroacetic acid solution from a 10 ml burette. The turbidity produced is then measured in a Klett Summerson

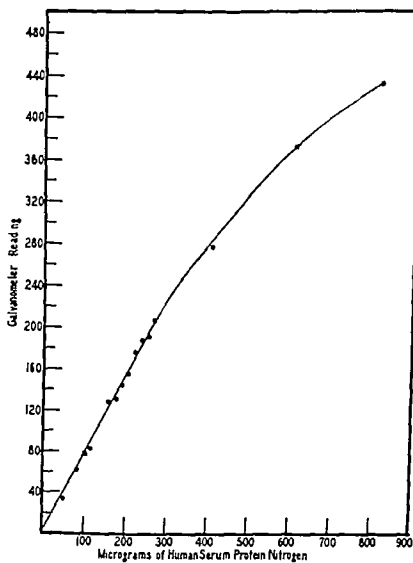


Fig 1 — A standard curve showing the relationship between turbidity readings and micrograms of serum protein nitrogen

tube after standing at least five minutes. The readings remain constant for more than two hours after precipitation. The amount of protein in the precipitates can then be estimated from a previously standardized curve which is obtained by measuring the turbidity of the trichloroacetic acid precipitate of a known amount of serum protein nitrogen. The results of a typical experiment are plotted in Fig 1. To substantiate that such a relationship is applicable to normal as well as abnormal serum we have compared the Kjeldahl nitrogen with the turbidity nitrogen.

*Determination of factor* Procure sample of fresh blood and determine oxyhemoglobin content by either the oxygen capacity or the carbon monoxide capacity method. Run a colorimetric determination on duplicate or triplicate 0.0 ml portions of this blood. From the known hemoglobin content of the blood the factor may be determined as follows:

$$\frac{\text{Gm per cent hemoglobin}}{\text{Colorimeter reading}} = \text{Oxyhemoglobin factor}$$

TABLE I RESULTS OF ANALYSES OF THE HEMOGLOBIN, ALBUMIN, AND GLOBULIN CONCENTRATIONS OF THIRTEEN INDIVIDUALS

NAME	HEMOGLOBIN		TOTAL PLASMA PROTEIN IN MG N/100					ALBUMIN NITROGEN				% ALBUMIN NITROGEN IN		% GLOBULIN DIFFER ENCE (K)
	GALVANOMETER READINGS (A)	(B)	KJELDAHL METHOD (C)	TURBIDITY (D)	% DEVIATION KJELDAHL METHOD (F)	N/MIL	TURBIDITY READING (±)	ALBUMIN N/SAMI (G)	N/MIL BLOOD (H)	KJELDAHL NITROGEN (I)	IN			
											TURBIDITY	(J)		
I H	436	16.2	6.80	6.53	-4.0		107-106	0.0290	2.96	44	45	55		
B F C	535	19.1	7.58	7.89	+4.1		106-104	0.0280	2.86	38	36	64		
I E	410	15.2	8.33	7.85	-5.8		103-103	0.0270	2.75	33	35	65		
I H	490	18.2	7.65	7.38	-3.5		109-106	0.0295	3.01	39	41	59		
C F	458	17.0	6.87	6.83	+0.6		104-103	0.0275	2.81	41	41	59		
J C	475	17.6	7.31	7.38	+0.1		88-90	0.0210	2.14	29	29	71		
I G	428	16.0	7.96	7.79	-2.1		93-95	0.0230	2.35	29	30	70		
L P	465	17.2	7.17	7.02	-2.1		106-104	0.0280	2.86	40	41	59		
S D	500	18.5	7.14	7.24	+1.4		98-102	0.0260	2.65	37	37	63		
L B	455	16.9	7.82	7.38	-5.6		107-106	0.0290	2.96	38	40	60		
P N	455	16.9	7.65	7.58	-0.1		97-96	0.0245	2.50	33	33	67		
C A	520	18.9	6.80	6.72	-0.1		102-99	0.0265	2.70	40	40	60		
R M	506	17.1	6.70	7.00	+4.5		101-100	0.0260	2.65	40	38	62		
Ave 17.0				Ave 7.00	Dev 2.6					Ave 37	Ave 37			
				Stand Dev 2.01										

The data (see Table II) are separated into four groups according to the percentage of albumin in the serum, ranging from 31 to 70 per cent. The amount of total serum nitrogen in each sample as determined by the Kjeldahl method is arbitrarily taken as 100 per cent with which the turbidity nitrogen is compared. The results demonstrate that the turbidity method gives a slightly higher result of less than 10 per cent and a standard deviation of about 5. Such an agreement between the two methods may be considered as satisfactory for clinical use. The results of a typical experiment with variations of the individual samples are recorded in columns C, D and E in Table I.

#### *Determination of Albumin Nitrogen—*

The amount of human albumin nitrogen in the supernatant (SU) is determined immunologically according to the procedure previously described. The following modifications are introduced in order to adapt this method to the small size of sample obtained by finger puncture. Two milliliters of the supernatant (SU) are diluted with an equal volume of 0.85 per cent NaCl solution. Two 1 ml samples of this solution are equivalent to 0.0008 ml of the original blood, are then added to two Klett Summerson tubes for high rate determinations. Four milliliters of the diluted antihuman albumin rabbit serum are added to each tube from a 50 ml burette, and the turbidity of the immune precipitate is measured after standing at room temperature for at least thirty minutes with a Klett Summerson photoelectric colorimeter using a blue filter of a wave length 420.

TABLE II DETERMINATION OF THE TOTAL PLASMA PROTEIN NITROGEN BY PRECIPITATION WITH TRICHLOROACETIC ACID

PER CENT OF ALBUMIN IN PLASMA	NUMBER OF SAMPLES	TOTAL PLASMA PROTEIN NITROGEN BY TURBIDITY METHOD*		
		%	±	S.D.
31.40	6	103	±	6.0
41.50	23	104	±	4.0
51.60	17	106	±	5.0
61.70	5	104	±	5.1

S.D. Standard deviation

\* Expressed as per cent of that found by the Kjeldahl method which is taken as 100 per cent

The amount of human albumin corresponding to the turbidity readings can be estimated from the standard curve. Multiplying the albumin content per sample (column G in Table I) by the serum dilution (i.e.  $51 \times 2$  equals 102) gives the total albumin nitrogen per milliliter of blood (column H). The ratio of total albumin nitrogen to the total nitrogen as determined either with the micro Kjeldahl method (column I) or from the turbidity of the trichloroacetic acid precipitate (column J), multiplied by 100 gives the per cent of the total nitrogen as albumin. The difference between 100 per cent and the per cent of albumin nitrogen gives the per cent of globulins (column K).

#### *Results—*

The results of a typical experiment with samples of blood drawn from thirteen individuals are summarized in Table I, in order to show the normal variations of routine determinations. They demonstrate that the agreement in total plasma protein nitrogen between the two methods (micro Kjeldahl and turbidity) is satisfactory for clinical use. The average percentage deviation of the turbidity nitrogen from the Kjeldahl nitrogen is 2.6 per cent. The same magnitude of deviation is reflected in the calculation of per cent of albumin based on total protein nitrogen measured with either one of the two methods. The average percentage albumin in the plasma of this group of individuals is only 37 per cent.

In Table III are summarized our data from thirty eight normal individuals. Similar results obtained by other leading methods are included for comparison. It can be noted that the average concentration of both hemoglobin and of plasma proteins in grams per 100 ml. of blood of our normal individuals is higher than that reported in the literature. Whether this difference can be attributed to the difference in the method of determination, or to the individual variation, is hard to decide on the basis of our limited number of experiments. Our average figures in the per cent of albumin in plasma approximate closely the reported figures of electrophoretic albumin,<sup>3</sup> but are significantly lower than those of methanol soluble albumin of Pillemer.

TABLE III SUMMARY OF DATA FROM NORMAL MEN ON HEMOGLOBIN, PLASMA PROTEINS, AND PER CENT ALBUMIN OBTAINED BY DIFFERENT METHODS

SUBSTANCE DETERMINED	METHODS USED	
	AS PRESENTED IN THIS PAPER	OTHER METHODS
Gm. hemoglobin in 100 ml. blood	16.0-21.5	(a) 14.1-17.2 <sup>1</sup> (b) 12.9-15.3 <sup>1</sup>
Gm. plasma proteins in 100 ml. blood	4.46 <sup>4</sup> 4.68 <sup>5</sup>	3.45-4.24
Per cent albumin in plasma	44.6 <sup>4</sup> 42.3 <sup>5</sup>	47 <sup>2</sup> 52 <sup>3</sup>

1 Copper sulfate method for measuring specific gravities of whole blood and plasma. R. A. Phillips, D. D. van Slyke, V. P. Dole, K. Emerson, Jr., P. B. Hamilton and R. M. Archibald. Published by Josiah Macy, Jr. Foundation, 1945, page 49.

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5 The immunologic method for the estimation of human albumin where the total nitrogen is determined by the turbidity method.

### *Comparison of Albumin Content in Venous and Capillary Blood—*

Although capillary blood may, under normal conditions, be used for the estimation of blood proteins, including hemoglobin and plasma, it may not serve as a representative sample of circulating blood in conditions of severe shock or in gross generalized edema. The former situation may cause the capillary to contain 30 to 40 per cent more cells and hemoglobin than the venous or arterial blood, and in the latter situation the edema fluid from subcutaneous tissue would exude with the blood from a puncture. It is believed that proper technique of finger or ear lobe puncture will minimize such errors.

In Table IV are collected the results on the comparison of per cent of albumin in plasma of capillary or venous blood of forty-three patients with various diseases, chiefly with malignant growth. In the tabulation of results it is arbitrarily assumed that the per cent of albumin in the plasma of the venous blood is 100, with which the albumin in the plasma of capillary blood is compared. The data are segregated into three groups of ascending per cent of albumin. The results demonstrate that the per cent albumin in the plasma of the capillary blood and the per cent in the plasma of venous blood agree with each other within the accuracy of the determination.

TABLE IV COMPARISON OF PER CENT OF ALBUMIN IN THE PLASMA OF CAPILLARY AND VENOUS BLOODS

PER CENT ALBUMIN	NUMBER OF SAMPLES	AVERAGE PER CENT ALBUMIN IN CAPILLARY BLOOD
30-35	3	34
36-45	26	36
46-55	14	102

The per cent of albumin in the venous blood is taken as 100 with which the per cent of albumin in the capillary blood is compared

## DISCUSSION

The pathologic conditions which can affect the plasma protein concentration and different types of anemia and polycythemia which can affect blood hemoglobin concentrations have been most adequately summarized by Kagan<sup>4</sup> and Phillips and associates. Hence, these will not be discussed in this report. It is generally agreed that so far as the determination of plasma protein concentration is concerned, an abnormal value is a definite proof that one of the physiologic conditions controlling the concentration has been disturbed. However a normal concentration cannot be regarded as an absence of any disturbed factor since determination of plasma protein concentration does not take into account a hemoconcentration or dilution.

It has been demonstrated that in animals<sup>5</sup> as well as in man<sup>6</sup> an outstanding change in plasma protein as a result of protein depletion due to either inadequate vitamin<sup>7</sup> protein or caloric intake or owing to a variety of diseases is a decrease in the albumin content even though the total plasma protein concentration may appear normal. It is therefore believed<sup>8, 9</sup> that the determination of the per cent albumin in plasma is of greater clinical significance than any change of plasma concentration. A procedure such as that we have just described should be useful not only for diagnostic purposes but also for field studies or public health work since it permits a rapid and accurate determination with a relatively small amount of sample.

## SUMMARY

A micromethod is described for the determination of hemoglobin, total plasma proteins, and albumin with 0.2 ml of capillary blood drawn by finger puncture. Blood is diluted with 0.85 per cent NaCl solution containing heparin. The suspension is centrifuged to remove red cells which are hemolyzed for the determination of its hemoglobin content by means of photoelectric colorimeter. The total protein nitrogen in the supernatant is estimated by measuring the turbidity of the trichloroacetic acid precipitate. The albumin nitrogen in the supernatant is determined immunologically. The difference between the total nitrogen and albumin nitrogen is taken as globulin nitrogen.

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## COLD HEMAGGLUTININ TEST BY A SLIDE METHOD

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THE cold hemagglutinin test recently has been widely used in the routine clinical study particularly in the diagnosis of primary atypical pneumonia. As far as we know, all previous workers have used the test tube method employed first by Landsteiner forty five years ago<sup>1</sup>. Recently Young<sup>2</sup> showed that the sensitivity of the test could be increased by observing the agglutination under the microscope by transferring a drop of cell suspensions to a plain glass slide. It occurred to us that the test might be performed on a hollow ground slide on which a direct microscopic examination could then be made. The results of the comparative study of the slide and the tube methods are herewith presented.

The preparation of the cell suspensions and the sera was carried out by the usual procedures described elsewhere.<sup>3</sup> A preliminary study on the relative sensitivity of group O erythrocytes from five donors including one of us (Hou) was made first. The results of the cold hemagglutinin test with eight sera from eight patients tested with cells of these donors were found approximately the same. Thereafter Hou's cells were used in nearly all of the tests reported in this paper.

### THE SLIDE METHOD

Special medicinal droppers (one drop approximately equivalent to 0.05 cc) and special microslides of 10 by 4 by 0.5 cm were used. On the surface of each slide were ten smoothly and evenly ground chambers in two rows measuring 1.5 cm in diameter and 0.2 cm in depth. In each of the ten chambers 1 drop of physiologic saline was placed and the same amount of the serum to be tested was then added to the first chamber. After the saline and the serum had been thoroughly mixed in the first chamber 1 drop of the mixture was transferred to the second chamber. Similarly twofold serial dilutions of the serum from 1:2 to 1:512 were made. No serum was added to the tenth chamber which served as a saline control. Finally 1 drop of 1 per cent cell suspensions was added to each of the ten chambers. The slide after being shaken was placed in the refrigerator at 0 to 4°C for two hours. At the end of this period the slide was taken out from the refrigerator, shaken gently and examined immediately under the microscope with a low power objective. The cell suspension showing barely visible agglutination was taken as the end point. Titers were recorded in terms of the final dilution of the serum multiplied by two. The specificity of the test was checked by the disappearance of the agglutination after the slide remained in an incubator at 37°C for one hour.

Aside from the regular slide method tests in which dilutions were first made in test tubes with pipettes and later performed on the slide were made simultaneously on sixty two specimens to determine the accuracy of the dilution by the dropper herein described. The following results were obtained. Thirty nine out of a total of sixty two specimens yielded the same titers by both methods of dilution. Except for one the specimens showed a difference in titers of not more than twofold dilution of these thirteen gave higher titers in tests with pipette dilution and nine in those with dropper dilution. The last one showed a higher titer in the test made with the dropper dilution (fourfold). It seems reasonable to conclude that the method of dilution with the dropper is accurate enough for practical use.

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Among the workers on cold hemagglutinin test there is a general agreement that it is best to examine the serum cell mixture by the tube method after refrigeration for fourteen to eighteen hours.<sup>2</sup> Therefore during the present study many tubes and slides were examined for agglutination after one, two, three, and eighteen hours of refrigeration at 0 to 4° C. Cold hemagglutinin titers nearly always reached the maximum at two hours for the slide method and eighteen hours for the tube method.

## RESULTS

During the period between May, 1946, and April, 1948, 509 blood specimens were collected from sixty-two normal individuals and 302 patients. Each of these specimens was tested according to the slide technique described. The tube test was simultaneously performed by Rose's method.<sup>3</sup> The results obtained are summarized in Tables I and II.

TABLE I CORRELATION OF COLD HEMAGGLUTININ TITERS BY SLIDE AND TUBE METHOD

SLIDE TUBE	NEG	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	TOTAL
Neg	26	33	65	48	20	1	0	0	0	0	193
1 4	1	2	17	33	17	3	0	0	0	0	73
1 8	2	1	22	31	29	9	0	0	0	0	94
1 16	0	1	1	11	22	15	1	0	0	0	51
1 32	0	1	0	5	15	15	7	1	0	0	44
1 64	0	0	0	0	2	6	17	3	0	0	28
1 128	0	0	0	0	1	0	6	5	3	0	15
1 256	0	0	0	0	0	1	1	4	1	1	8
1 512	0	0	0	0	0	0	1	0	1	0	2
1 1024	0	0	0	0	0	0	0	0	0	1	1
Total	29	38	105	128	106	50	33	13	5	2	509

Table I shows the correlation of the cold hemagglutinin titers between the slide and the tube methods. It is obvious that the slide method is more sensitive than the tube method. There were only ninety-four (18.5 per cent) specimens whose cold hemagglutinin titers were the same by both methods, and eighteen (3.5 per cent) specimens whose cold hemagglutinin titers by slide method were

TABLE II DISTRIBUTION OF MAXIMUM TITERS OF COLD HEMAGGLUTININS BY SLIDE METHOD IN 364 SUBJECTS

CLINICAL GROUP	COLD HEMAGGLUTININ TITERS										TOTAL
	NEG	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	
Normal individual	1	6	16	21	14	4	0	0	0	0	56
Primary atypical pneumonia	0	0	0	0	0	1	5	4	3	2	15
Lobar pneumonia	1	3	4	4	11	5	0	0	0	0	28
Pulmonary tuberculosis	2	2	6	13	11	0	2	0	0	0	36
Lung abscess	0	1	3	2	2	1	0	0	0	0	9
Other respiratory diseases	0	3	6	3	6	4	0	1	0	0	23
The pleurisy and peritonitis	0	1	3	8	3	3	1	0	0	0	19
Kala azar	11	3	6	23	19	8	9	1	1	0	81
Typhus fever	0	0	3	2	1	3	0	0	0	0	9
Typhoid fever	0	0	1	0	3	1	0	0	0	0	5
Acute epidemic encephalitis	1	1	2	6	2	2	1	0	0	0	15
Acute epidemic hepatitis	1	3	0	0	1	0	0	0	0	0	5
Cirrhosis of liver	2	2	4	5	11	2	7	1	0	0	34
Anemia	0	0	4	2	3	3	1	0	0	0	13
Bright's disease	0	0	1	3	1	0	0	0	0	0	5
Cancer	0	0	2	2	1	0	0	0	0	0	5

lower than those by the tube method. For the remaining 397 (78 per cent) specimens the slide method gave higher titers: twofold dilution 141 (27.7 per cent), fourfold dilution, 156 (30.6 per cent), eightfold dilution 76 (15 per cent), and sixteenfold dilution, 24 (4.7 per cent).

From Table II it seems clear that high titers were rare except in primary atypical pneumonia, while low titers were common in various diseases as well as in normal controls. The highest titer of cold hemagglutinins obtained in our sixty-two normal individuals was 1:64. Therefore in the present study only titers above 1:64 were considered significant.

## DISCUSSION

From the foregoing study the slide method is shown to be more sensitive than the tube method. It is to be noted that the staining of the cell and serum used in the present method is practically identical with that of the tube method. The difference in the sensitivity of the two methods is shown by the results presented in Table III can be explained only by the difference in the method of examining agglutination. The exact mechanism of this phenomenon is not yet clear.

Despite the sensitiveness of the slide method high titers of cold hemagglutinins were still uncommon except in primary atypical pneumonia (93.3 per cent of fifteen cases). But low titers occurred more frequently in various pathologic and normal sera. It is of some interest that 23.5 per cent of thirty-four cases of cirrhosis of the liver and 13.6 per cent of eleven cases of kala-azar showed significantly high titers of cold hemagglutinins.

TABLE III. COMPARISON OF SLIDE AND TUBE COLD HEMAGGLUTININ TITERS BY DIFFERENT METHODS OF EXAMINATION

SERA	SLIDE TITERS	TUBE TITERS			
		2 HR.		18 HR.	
		GROSSLY	MICROSCOPICALLY	GROSSLY	MICROSCOPICALLY
1	1:32	0	1:4	1:4	1:16
2	1:32	0	1:4	1:4	1:8
3	1:16	0	1:4	1:4	1:16
4	1:16	0	0	0	1:4
5	1:32	0	0	0	0
6	1:64	0	1:4	0	1:4
7	1:16	0	1:4	0	1:4
8	1:16	0	1:4	1:4	1:8
9	1:32	0	0	0	1:4
10	0	0	0	0	0

## SUMMARY

A slide method for the determination of cold hemagglutinin titer has been described which is not only more rapid but also simpler than the tube method. It does not require tubes and serologic pipettes. In a very small hospital laboratory it might be advantageous to escape the necessity of washing tubes and to be able to start another series of tests after merely rinsing off the slide.

## REFERENCES

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- Rose, H. M. Cold Hemagglutinins in Visceral Leishmaniasis. *Proc. Soc. Exper. Med.* 58: 93, 1945.

Among the workers on cold hemagglutinin test there is a general agreement that it is best to examine the serum cell mixture by the tube method after refrigeration for fourteen to eighteen hours.<sup>2</sup> Therefore during the present study many tubes and slides were examined for agglutination after one, two, three, and eighteen hours of refrigeration at 0 to 4° C. Cold hemagglutinin titers nearly always reached the maximum at two hours for the slide method and eighteen hours for the tube method.

## RESULTS

During the period between May, 1946, and April, 1948, 509 blood specimens were collected from sixty-two normal individuals and 302 patients. Each of these specimens was tested according to the slide technique described. The tube test was simultaneously performed by Rose's method.<sup>3</sup> The results obtained are summarized in Tables I and II.

TABLE I CORRELATION OF COLD HEMAGGLUTININ TITERS BY SLIDE AND TUBE METHOD

SLIDE 1 UBF	NEG	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	TOTAL
Neg	26	33	65	48	20	1	0	0	0	0	193
1 4	1	2	17	33	17	3	0	0	0	0	73
1 8	2	1	22	31	29	9	0	0	0	0	94
1 16	0	1	1	11	22	15	1	0	0	0	51
1 32	0	1	0	5	15	15	7	1	0	0	44
1 64	0	0	0	0	2	6	17	3	0	0	28
1 128	0	0	0	0	1	0	6	5	3	0	19
1 256	0	0	0	0	0	1	1	4	1	1	8
1 512	0	0	0	0	0	0	1	0	1	0	2
1 1024	0	0	0	0	0	0	0	0	0	1	1
Total	29	38	105	128	106	50	33	13	5	2	509

Table I shows the correlation of the cold hemagglutinin titers between the slide and the tube methods. It is obvious that the slide method is more sensitive than the tube method. There were only ninety-four (18.5 per cent) specimens whose cold hemagglutinin titers were the same by both methods, and eighteen (3.5 per cent) specimens whose cold hemagglutinin titers by slide method were

TABLE II DISTRIBUTION OF MAXIMUM TITERS OF COLD HEMAGGLUTININS BY SLIDE METHOD IN 304 SUBJECTS

CLINICAL GROUP	COLD HEMAGGLUTININ TITERS										TOTAL
	NEG	1 4	1 8	1 16	1 32	1 64	1 128	1 256	1 512	1 1024	
Normal individual	1	6	16	21	14	4	0	0	0	0	1
Primary atypical pneumonia	0	0	0	0	0	1	5	4	3	0	
Lobar pneumonia	1	3	4	4	11	5	0	0	0	0	
Pulmonary tuberculosis	2	2	6	13	11	0	2	0	0	0	
Lung abscess	0	1	3	2	2	1	0	0	0	0	
Other respiratory diseases	0	3	6	3	6	4	0	1	0	0	
The pleurisy and peritonitis	0	1	3	8	3	3	1	0	0	0	
Kala azar	11	3	6	23	19	8	9	1	1	0	
Typhus fever	0	0	3	2	1	3	0	0	0	0	
Typhoid fever	0	0	1	0	3	1	0	0	0	0	
Acute epidemic encephalitis	1	1	2	6	2	2	1	0	0	0	
Acute epidemic hepatitis	1	3	0	0	1	0	0	0	0	0	
Cirrhosis of liver	2	2	4	5	11	2	7	1	0	0	
Anemia	0	0	4	2	3	3	1	0	0	0	
Bright's disease	0	0	1	3	1	0	0	0	0	0	
Cancer	0	0	2	2	1	0	0	0	0	0	

reducing valve controls the pressure in the tank and therefore in the cuff during its inflated phase, this is set at around 5 pounds pressure which is above arterial cutoff but is not sufficient to cause local pressure pain

Transition from an inflated cuff to a totally deflated one and vice versa occurs in approximately one fifth second. This results in very little pooling of blood in the limb, which occurs markedly when the cuff is inflated to any point below systolic pressure for a long period of time

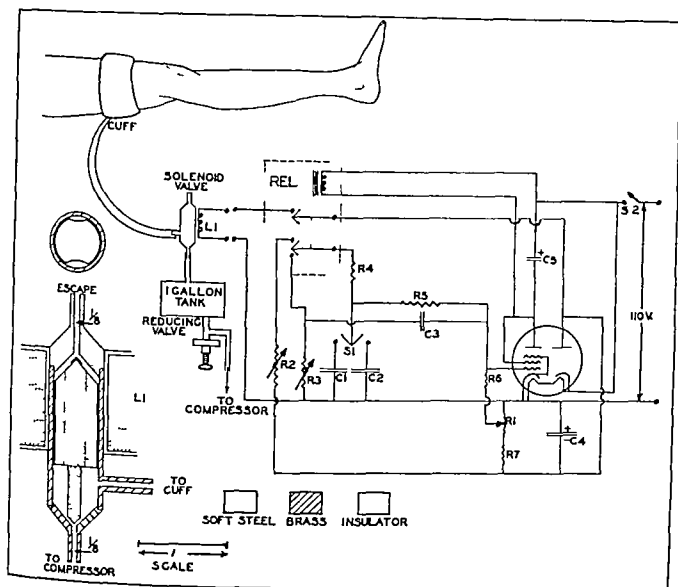


Fig 1—Electrical diagram and mechanical drawing of the apparatus for producing quantitative ischemia.

L1 Solenoid valve coil (3 400 turns #34 wire)  
REL Double pole double throw plate relay resistance 500 ohms  
S1 Single pole double throw toggle switch  
S2 Single pole single throw toggle switch  
R1 0 000 ohm, wirewound potentiometer  
R2 and R3 9 megohm carbon potentiometers log taper  
R4 50 000 ohm  $\frac{1}{2}$  watt resistor

R5 40 megohm  $\frac{1}{2}$  watt resistor  
R6 0 megohm  $\frac{1}{2}$  watt resistor  
R7 100 000 ohm 1 watt resistor  
C1 0 1 mfd. 00 volt paper condenser  
C2 10 mfd. 00 volt paper condenser  
C3 0 000 5 mfd. 200 volt paper condenser  
C4 and C5 8 mfd. 175 volt, electrolytic condensers  
Tube 117L7/A17 GT

Using this apparatus one can state in percentage the amount of time that the blood flows, and various states of ischemia can be reproduced. During the phase of blood flow there is a compensatory vasodilatation which appears to be four to six times normal. For this reason significant ischemia does not result until the period of zero blood flow is approximately 80 per cent of the cycle. When this is increased to 95 per cent severe pain ensues both in man and in

dogs Dogs in the second stage of Nembutal anesthesia will unconsciously wail loudly when this phase of ischemia is attained and will cease wailing as soon as the ischemia is lessened Such results may be repeated within narrow limits The reactions of animals to ischemia will be further analyzed in experiments designed to elucidate the cause of pain in Buerger's disease, Raynaud's disease, immersion foot, and acute poliomyelitis

### Erratum

In the article by LeRoy and Nulefski, "Dicumuirol in Experimental Myocardial Infarction," in the August, 1948, issue of the JOURNAL, Table I should read as follows

PROTHROMBIN TIME (SEC.)	PER CENT PROTHROMBIN TIME
6	100%
7.5	80%
10	60%
12	50%
15	40%
20	30%
30	20%

# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty First Annual Meeting

Chicago, Ill., Oct 29 and 30 1948

## PROGRAM

SCIENTIFIC PROGRAM—OCTOBER 29 1948

FRIDAY MORNING, 9 15 A M

### 1 A STUDY OF PULMONARY HYPERTENSION IN CHRONIC HYPERTROPHIC PULMONARY EMPHYSEMA

CRAIG BORDEN, M.D., RUSSELL H. WILSON, M.D. AND RICHARD A. LEBERT, M.D.  
MINNEAPOLIS, MINN.

(INTRODUCED BY CECIL JAMES WATSON, M.D.)

For many years the presence of pulmonary hypertension in chronic hypertrophic pulmonary emphysema has been suspected because of the occurrence of right heart failure, the presence of right ventricular hypertrophy at autopsy and electrocardiographic changes interpreted as indicative of right heart strain. Recently, the development of venous catheterization and the use of high frequency manometers have permitted the accurate measurement of pulmonary arterial pressure in patients with this disease.

In fifteen patients with chronic hypertrophic pulmonary emphysema the average pressure in the pulmonary artery was  $33.9 \pm 10.4$  mm Hg systolic and  $20.9 \pm 4.7$  mm Hg diastolic. This is compared with an average pressure of  $20.1 \pm 3.7$  mm Hg systolic and  $8.8 \pm 1.5$  mm Hg diastolic in twelve normal subjects. The mean cardiac index in the patients with emphysema was  $3.38 \pm 0.67$  liters per minute and  $3.59 \pm 0.72$  liters per minute in the normal subjects. Inasmuch as there is no reason to believe that the left auricular pressure is elevated in emphysema, this study indicates that the resistance in the pulmonary vascular bed is increased. The abnormally high intrapleural pressure which has been described in emphysema would account for a small portion of the rise in pulmonary arterial pressure.

A striking feature of the pulmonary arterial pressure in emphysema is the respiratory variation characterized by a marked decrease in pressure at the beginning of inspiration.

The severity of the emphysema was evaluated by measurements of vital capacity, ratio of residual air to total lung volume, pulmonary emptying rate and the degree of oxygen unsaturation of arterial blood. There was no definite correlation between the degree of pulmonary hypertension and the severity of the emphysema as estimated by these measurements. The development of pulmonary hypertension does not appear to be completely dependent upon those factors which result in the loss of pulmonary function.

Four of the fifteen patients had definite evidence of right heart failure manifested by peripheral edema, hepatomegaly and a markedly elevated venous pressure. In these four patients the degree of pulmonary hypertension was not significantly different from those without right heart failure. The development of cor pulmonale and right heart failure in pulmonary emphysema does not appear to be a consequence solely of the pulmonary hypertension but probably involves other factors such as chronic anoxia.

## 2 STUDIES ON CARDIORESPIRATORY FUNCTION WITH THE OXYHEMOGRAPH

BEN E GOODRICH, M D, VIVIAN G BEHRMANN, PH D (BY INVITATION), AND  
FRANK W HARTMAN, M D (BY INVITATION), DETROIT, MICH

Preliminary studies with the oxyhemograph have been conducted on patients subject to varying degrees of dyspnea resulting from cardiac or respiratory disabilities. Young, apparently healthy adults also were tested.

Continuous recording of the oxygen saturation of circulating hemoglobin was afforded through the use of the oxyhemograph described by Hartman, Behrmann, and Chapman. The initial arterial oxygen saturation at rest was calculated from calibration after the patient produced a stabilized level while breathing 100 per cent oxygen. After standardization, the subjects were tested in atmospheric oxygen by simple exercises of breath holding and knee bending while supine and erect. The resultant tracings were charted for comparison using arbitrary time intervals and percentage levels.

Results of these tests reveal differences in the degree and duration of changes in arterial oxygenation in various individuals. Healthy adults, asymptomatic in daily life, varied greatly in the extent of lessened oxygenation possible by voluntary breath holding. Well adults showed moderate variation in the per cent increase in the blood oxygen saturation concomitant with exercise, although cessation of exercise uniformly resulted in an immediate depression of oxyhemoglobin to or below the atmospheric level. The return to normal was prompt. The exercise was not of a degree that exhausted the subject. In patients under similar tests exercise was not associated with increased oxyhemoglobin. Patients often exhibited marked hypoxemia with a delayed recovery. Not infrequently recovery was stamptep-like in appearance.

These findings indicate that both the degree of arterial unsaturation and the character of the recovery curve may be of clinical significance. The post exercise curve may vary as to whether recovery is spontaneous or aided by the administration of 100 per cent oxygen. Charts present typical oxyhemograms. Bronchospastic dyspnea is contrasted to structural respiratory disability. Patients with decreased cardiac reserve show abnormal curves. In these patients abnormal curves may be recorded even though symptoms are denied.

These preliminary studies indicate that the oxyhemograph together with simple tests may prove useful in the differential clinical evaluation of cardiorespiratory function.

## 3 THE PHYSICAL MECHANISM OF FIBROTIC REACTIONS

SILAS M EVANS, M D, AND WALTER ZEIT, PH D, MILWAUKEE, WIS

(INTRODUCED BY WILLIAM S MIDDLETON, M D)

Quartz, cholesterol, and tuberculosis produce fibrous reactions in tissue which differ quantitatively but not qualitatively in histology. This has been demonstrated experimentally by others. The symbiotic relationships between quartz and tuberculosis phospholipid have been repeatedly demonstrated.

Fibrous tissue reactions are currently considered as protective against toxins and poisons in solutions. It is pointed out that fibrous tissue is unqualified physiologically as a barrier against substances in solution. Fibrous elements are adapted to confine stress in the physical sense. In health and disease, fibrosis contributes physical support to the biologic economy.



The architecture of fibrous tissue reaction conforms with Newton's equations of force. This is demonstrated by slides of biologic preparations compared with force field preparations using magnets and non fibrous

Many irreconcilable objections are recognized by those who have reviewed the solubility theory of silicosis. These are enumerated. These objections are reconcilable with another basic nonchemical mechanism.

A search for physical properties common to fibrosive materials has revealed that there is a direct relationship between the fibrosive properties of a substance and its solid state molecular symmetry. Detailed reports are in press at the present time elaborating this point.

Those substances which exist in a state of molecular asymmetry compatible with piezoelectric reactions are observed to produce fibrosis. Piezoelectricity is a mechanism whereby mechanical and electrical energy states may be converted from one to the other in either direction. A more detailed definition of piezoelectricity is presented.

One phase of our present study is presented. Barium titanate was selected because of its spectacular piezoelectric properties, its lack of solubility, and the absence of elemental silicon in its chemical makeup. The preparation of animals is described and the results obtained are presented with the use of slides and tables.

A reference is made to the scope of the present problem and reports now in press.

*Conclusions* —(1) Fibrosive properties and piezoelectric properties of materials introduced into tissues are directly correlated.

2 The fibrosive potential of a material selected on the basis of its physical properties alone has been demonstrated.

#### 4 THE EFFECT OF THE ANTICOAGULANT DRUGS UPON THE CORONARY FLOW

N. C. GILBERT, M.D. AND LESTER NALEFSKI, M.D. (BY INVITATION)  
CHICAGO, ILL.

During the last few years the anticoagulant drugs, heparin and Dicumarol, have been shown to have a favorable effect upon coronary occlusion. Mortality has been greatly reduced and peripheral vascular accidents have been made much less frequent through the use of these drugs. It does not seem altogether reasonable, however, that these results are due to the anticoagulant action of these drugs alone. When the case reaches the attention of the physician, the occlusion and infarct are already an accomplished fact. Anticoagulant therapy cannot alter the already formed thrombus whatever effect it might have upon future thrombotic processes. There is no definite evidence that the clot propagates or extends, except on rare occasions and there is a great deal of evidence that the clot does not propagate. It has been shown also that many instances of occlusion are initiated by a subintimal hemorrhage. According to Wartman, these latter comprise at least 18 per cent.

Our own clinical results with coronary vasodilator drugs such as aminophylline followed by oral preparations of the xanthines, closely parallel the results obtained by others with anticoagulant therapy.

In experimental work upon animals and upon the empty beating heart we have been able to show that the use of either heparin or Dicumarol increases the coronary flow volume. With heparin the effect varies from no effect to

a moderate effect, depending upon the preparation. Dicumaiol, however, has a very definite effect upon the coronary flow. The effect is equal to that of the xanthines, and apparently lasts longer. We believe that the favorable effects resulting from the use of these drugs are primarily due to their action in increasing the coronary flow.

## 5 CORRELATION OF ELECTROCARDIOGRAPHIC AND PATHOLOGIC FINDINGS IN INFARCTION OF THE INTERVENTRICULAR SEPTUM

GORDON B. MYERS, M.D., HOWARD A. KLEIN, M.D. (BY INVITATION), AND  
TOMIHARU HIRATZKA, M.D. (BY INVITATION), DETROIT, MICH.

In a consecutive series of 161 patients with pathologically established myocardial infarction with Wilson precordial, standard and Goldberger limb leads, infarction of the interventricular septum was demonstrated in 102 patients and was accurately localized by means of coronary injection with radiopaque mass, roentgenogram, and multiple microscopic blocks. The cases were classified into three groups according to distribution of the pathologic lesion: (1) infarction largely confined to the septum, nine cases, (2) septal extension of anterior or anteroposterior infarction, sixty-nine cases, (3) septal extension of posterior infarction, twenty-four cases.

The electrocardiogram furnished suggestive or diagnostic evidence of septal infarction in all patients in group 1, in 80 per cent of group 2, and in only 33 per cent of group 3. This evidence was derived from the precordial leads in all but three instances in which it was obtained from  $aV_F$ . The standard leads were of no help. Diagnostic failures were prone to occur when the septal portion of the infarct was limited to the posterior half or to the apical third of the septum. The electrocardiographic findings were classified as follows:

1 Complete auriculoventricular block due to acute posteroseptal infarct was present in two patients.

2 QRS-T abnormalities diagnostic of infarction of the anterior portion of the septum were found in eight precordial leads of forty patients. These comprised (1) right bundle branch characterized by an abnormal Q wave and/or elevated RS-T junction in  $V_{1-2}$  in thirteen patients, (2) triphasic qRS complex of normal duration in  $V_{1-2}$  in five patients, (3) QS complexes and upward RS-T displacement in leads over the right atrium, accompanied by abnormal Q waves in leads farther to the left, in twenty-two patients. QRS-T abnormalities in  $V_{1-2}$  that were considered suggestive, but not diagnostic, of septal infarction were found in eighteen other instances. An abnormal QS or qRS complex referable to infarction of the posterior portion of the septum was recorded in lead  $aV_F$  as a result of horizontal cardiac position in seven patients.

3 Left bundle branch block was present in four instances, but was attributable to septal infarction in only one. Patterns characterized by QRS prolongation, an initial downstroke, and late intrinsicoid deflection in left axillary leads were present in ten patients, but were ascribed to subendocardial anterolateral infarction rather than the septal lesion.

4 Signs of anteroposterior infarction, considered presumptive of involvement of the intervening septum, were present in nine additional patients.

The correlation of electrocardiographic and pathologic findings will be brought out by case presentations.

## 6 BEDPAN DEATHS

JOHNSON MCGUIRL, M D, ROBERT GREEN M D (BY INVITATION),  
 SANFORD R. COURTER M D (BY INVITATION) JEAN NOERTKER A B  
 (BY INVITATION), ARNOLD IGLAUER, M D VIRGIL HAUENSTEIN, M D AND  
 JOHN BRAUNSTEIN, M D (BY INVITATION), CINCINNATI OHIO

The notorious frequency of sudden and unexpected deaths of patients while using bedpans in hospitals has been commented upon for many years

In an attempt to discover the factors responsible for such deaths the autopsy protocols and clinical records of patients in the Cincinnati General Hospital who died on bedpans and were autopsied have been reviewed for the ten year period from 1936 to 1946, inclusive

A second approach to the problem of determining the causes of death in such cases has been the measurement of alterations in circulatory dynamics accompanying simulated efforts of normal individuals and patients with organic heart disease to move the bowels

The precise mechanism of death in the twelve cases examined at post mortem (all with organic heart disease) was determined in only three instances (1) ruptured aortic valve (2) pulmonary embolism (3) ruptured dissecting aneurysm

Intravascular pressures measured by strain gauges and graphically recorded in normal subjects and patients demonstrated marked rises of pressure during the initial phase of bearing down within the femoral artery left ventricle pulmonary artery, right ventricle right auricle and peripheral veins Toward the end of the strain a fall in blood pressure occurred subsequently a marked rise of pressure developed

Electrocardiographic tracings in twenty five normal subjects during strain showed first bradycardia then marked tachycardia Following cessation of strain, marked bradycardia developed Migration of the pacemaker and premature contractions occasionally were noted Patients with organic cardiovascular disease showed few such changes

Since the cause of death frequently is not found at autopsy it is believed that cardiac arrhythmias or cardiac standstill may be the usual cause of bed pan deaths

Measures to combat such deaths are suggested

## 7 EMETINE THERAPY IN THE PRESENCE OF HEART DISEASE

W A SODEMAN M D NEW ORLEANS LA

Electrocardiographic changes occur in 25 to 50 per cent of patients with normal hearts under emetine therapy and the drug is generally considered contraindicated in the presence of heart disease We have studied eight patients in whom advanced heart disease was present and to whom emetine was administered because hepatic amebiasis was a greater threat to life than was the heart disease In five closed drainage also was performed All were at bed rest Five had arteriosclerotic heart disease, two being at the time under digitalis therapy One had in addition, hypertension and known previous congestive heart failure One had congenital heart disease with interventricular septal defect and bundle branch block, another rheumatic heart disease with mitral stenosis Six showed abnormal electrocardiograms before emetine therapy was started Under a controlled regimen of treatment with emetine dosage based upon body weight (1 mg per kilogram body weight daily not to exceed ten days) none of the pa

tients developed symptoms or signs of heart failure. Only one showed additional electrocardiographic changes attributable to emetine. One death occurred three months and another one year following therapy, both from other known causes. In all patients the amebiasis was effectively controlled.

Despite usual warnings that emetine should not be administered to cardiac patients, dangers from hepatic amebic disease at times outweigh those that may result to the heart from emetine and treatment should be instituted. The added risk in the use of emetine under these circumstances, when its need is great, must be accepted. Adjustment of dosage schedules to fall within 1 mg per kilogram body weight daily for ten days was, in the eight patients cited, effective for the amebiasis and was accompanied by no demonstrable adverse effects on the already damaged cardiovascular system. The use of emetine in the presence of heart disease is discouraged except when necessary for forms of amebiasis endangering the patient's life and not effectively treated otherwise.

## 8 THE PRESIDENT'S ADDRESS

### 9 THE PATHOGENESIS OF ACUTE BACTERIAL LYMPHADENITIS

RALPH O. SMITH, M.D. (BY INVITATION), AND W. BARRY WOOD, JR., M.D.  
St. Louis, Mo.

In an effort to clarify the role of lymphatic tissue in antibacterial defense, a study has been made of the cellular reactions in popliteal lymph nodes of rats inoculated in the foot pads with Type 1 pneumococcus.

Acute pneumococcal lymphadenitis is characterized by rapid infiltration of polymorphonuclear leucocytes into the intermediary sinuses of the node and prompt phagocytosis by both the macrophages of the sinuses and the recently arrived leucocytes. By seven hours the polymorphonuclear leucocytes are found densely congregated about the hilar region, and nine hours after inoculation the phagocytosed organisms have been digested and pneumococci are no longer seen in the node. At the end of twenty-four hours the node presents the picture of a subsiding inflammation with a marked macrophage reaction and regenerating follicles.

By eradication of hilar blood supply, direct intralymphatic injection of pneumococci, and analysis of cells in afferent lymph, it has been shown that the majority of polymorphonuclear leucocytes entering the intermediary sinuses come from capillaries lining these sinuses, whereas the leucocytes present in the subcapsular sinus come from the primary inflammatory focus in the footpad as well as from capillaries of the capsule and the subcapsular portions of the follicles.

Phagocytosis of pneumococci in the foot pad and popliteal node occurs in less than thirty minutes after inoculation. Because of the promptness with which the phagocytic reaction takes place, and because of the large surface area afforded the leucocytes by the nodal sinuses and interstitial tissues of the foot pad, it is assumed that the same nonantibody mechanism of "surface phagocytosis" is involved as that previously described in experimental pneumonia.

Fibrin formation in the sinuses of the node is rare. This finding may be related to the observation that five minutes after inoculation, mast cell granules, which are known to contain heparin, are strewn throughout the sinuses of the node. The mast cells become vacuolated and almost devoid of granules, later their granules appear to regenerate.

These studies indicate that, early in the course of acute infection, bacteria reaching a regional lymph node elicit a prompt leucocytic response in the node.

The resulting enhancement of filtration due to the congregation of leucocytes in the hilar sinuses and the immediate phagocytosis of bacteria in the absence of opsonin lead to rapid destruction of invading organisms. Thus an acutely inflamed lymph node, so frequently regarded as a passive filter, in reality plays an active role in antibacterial defense.

## 10 ANTIBODY FORMATION IN HUMAN SUBJECTS FOLLOWING THE INGESTION OF HEAT KILLED BRUCELLA ABORTUS

ABRAHAM I. BRAUDE, M.D., and DAVID GOLD, B.S., MINNEAPOLIS, MINN.

(INTRODUCED BY WESLEY W. SPINK, M.D.)

The agglutination test is used widely in the diagnosis of brucellosis. Caution must be exercised in its interpretation however because agglutinins are known to appear in the sera of persons without brucellosis. Eisele and McCullough have found that positive *Brucella* agglutination tests develop following cholera vaccination. This probably accounts for only a small percentage of false positive reactions. At the University Hospitals Aagaard has observed that *Brucella* agglutinins were present in 93 per cent of blood samples obtained from consecutive rural patients who were being studied in a dispensary service for various cases. The following investigation was undertaken to determine whether the repeated ingestion of nonviable *Brucella* organisms in milk would produce demonstrable agglutinins in the serum.

A preliminary study disclosed that no agglutinins appeared in the sera of guinea pigs following the oral administration of 100 billion heat killed *Brucella abortus* organisms in single or divided dosages. The same dose was then given in aqueous suspension to twenty three human volunteers in a single feeding and it was found that serum agglutinins had developed in four subjects. An attempt was made next to approximate the naturally occurring circumstances whereby *Brucella* enter the gastrointestinal tract in pasteurized milk. This was accomplished by the daily feeding to thirty six hospital patients of 100 million heat killed *Brucella* organisms in pasteurized milk obtained from *Brucella* free herds. After ten to twenty feedings during periods of two to four weeks significant titers of agglutinins were detected in eleven of the thirty six persons. When the total number of ingested *Brucella* was reduced to one million daily, it was found that one of eight additional patients showed a rise in titer. In a total of sixty nine individuals agglutinins were produced in sixteen following the ingestion of nonviable *Brucella* under these varying conditions. Titers ranged from 1:20 to 1:640.

The ability of dead *Brucella* to sensitize the skin to *Brucella* antigens was investigated in eighteen persons who ingested 100 million organisms daily for ten to twenty feedings. Sixteen of the eighteen had negative skin tests with *Brucellergen* antigen after the feedings were discontinued. Two persons with positive tests at the beginning of the experiment showed no alteration in skin sensitivity as a result of the feedings. The sera of thirteen subjects in whom agglutinins for *Brucella* had appeared were also tested for opsonins before and after the ingestion of *Brucella*. In seven of these there was an increase of ten to ninety over the initial opsonocytaphagic index.

From these results it is concluded that the oral ingestion of nonviable *Brucella* may give rise to the production of agglutinins or opsonins but not to the acquisition of dermal hypersensitivity. This is offered as one explanation for the fairly high incidence of agglutinins in the blood of asymptomatic persons who inhabit areas where Bang's disease is prevalent in cattle. Under naturally

occurring conditions these dead organisms may be ingested in pasteurized milk or may result from the action of gastric juice on living organisms which enter the stomach in raw milk. Data have been accumulated demonstrating the marked anti-*Brucella* action of human gastric juice.

## 11 THE DEVELOPMENT OF STREPTOMYCIN-RESISTANT BACTERIA IN THE STOOLS OF PATIENTS TREATED FOR TUBERCULOSIS

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The development of streptomycin-resistant pathogens during streptomycin therapy has been demonstrated often enough to establish this phenomenon as a serious therapeutic and public health risk. However, no studies have been made of the effect of streptomycin therapy on bacteria which normally lead a saprophytic existence in the body but which are nevertheless potentially pathogenic. To investigate this problem, studies have been made of the streptomycin resistance of coliform bacilli in the stools of patients given streptomycin for various types of tuberculosis.

Samples of stool were obtained when possible prior to therapy, and at various intervals during and after therapy. Saline suspensions of the stool were streaked on each of two plates: an eosin-methylene blue plate and a similar plate containing 100  $\mu$ g streptomycin per cubic centimeter. Growth was recorded after twenty-four, forty-eight, and seventy-two hours of incubation, photographs of the plates were made, and representative colonies were picked for more accurate determination of streptomycin resistance in a serial dilution test.

The study included seven patients with milary tuberculosis, eleven with nonmilary pulmonary tuberculosis, six with tuberculous peritonitis and three with tuberculous pericarditis. All received 1 or 2 Gm of streptomycin daily intramuscularly for one to twelve weeks, but no streptomycin by any other route.

Colon or aerogenes bacilli which grew freely in 156 to more than 2,500  $\mu$ g streptomycin per cubic centimeter of medium appeared during treatment in the stools of six out of seven patients with milary tuberculosis and in four of six patients with tuberculous peritonitis, but in no case of pericarditis and in only one of eleven cases of nonmilary pulmonary tuberculosis. Sensitive strains from these patients were inhibited by 0.6 to 2.4  $\mu$ g per cubic centimeter.

The ecologic relationship in the stool between sensitive and resistant variants was interesting. Though sometimes the resistant forms entirely replaced the sensitive strains during treatment, sensitive bacilli returned after cessation of therapy and usually ultimately completely replaced the resistant forms. This phenomenon was not a reversion of resistant to sensitive variants, nor a manifestation of "streptomycin-dependency," because resistant strains retained their resistance after more than 100 passages in streptomycin free broth and after twelve passages through untreated mice.

In two patients with tuberculous peritonitis, resistant bacilli decreased in number or disappeared during the stage of clinical improvement while the patients were still receiving streptomycin.

# 12 BACTEREMIA FOLLOWING TOOTH EXTRACTION PREVENTION WITH PENICILLIN AND 3,4-DIMETHYL-5-SULFANILAMIDE ISOXAZOLE (GANTROSAN)

PAUL S. RHODES, M.D., and WARREN R. SCHRAM, D.D.S. (BY INVITATION)  
CHICAGO, ILL.

WITH THE TECHNICAL ASSISTANCE OF DORIS ADAIR

The present study was undertaken with the following objectives: (1) To confirm the contention of other investigators that a temporary bacteremia can be demonstrated in a fairly high percentage of persons immediately after tooth extractions; (2) To learn if possible which teeth are most likely to cause bacteremia when extracted or traumatized; (3) To determine the effectiveness of penicillin and a new sulfonamide, 3,4-dimethyl-5-sulfanilamide isoxazole (Gantrosan), in preventing the blood stream dissemination of bacteria.

One hundred twenty-two blood cultures were done immediately after teeth were extracted either because the teeth were infected or to prepare the gums for full dentures. In a group of twenty-eight the patients received intramuscular procaine penicillin in oil (300,000 units) two hours before extraction and the blood culture was positive in two (7.2 per cent). In a group of eight patients receiving 100,000 units of penicillin in aqueous solution two hours before tooth extraction there were no positive blood cultures. Eighteen blood cultures were from patients who received 2 Gm. of Gantrosan orally two hours before tooth extraction and none of these were positive. Thus in the entire protected group the incidence of temporary bacteremia was 3.7 per cent. In the control group of sixty-eight in which the patients received no preoperative medication 35.3 per cent of the blood cultures were positive for nonhemolytic streptococci.

Quantitative cultures were made of the snapped off roots of the teeth extracted. The bacterial counts of the teeth removed in the protected and nonprotected groups were approximately the same. In attempting to correlate the roentgen appearance of the teeth with bacterial counts of the root ends and incidence of postoperative positive blood cultures, no constant relationship could be determined. Frankly abscessed teeth were associated with post extraction bacteremia a little more often than roentgenographically negative teeth, but in many instances bacteremia resulted from extraction of the latter also.

The extreme importance of protecting persons with known valvular heart disease with penicillin or sulfonamide before tooth extractions is obvious. To be on the safe side the procedure should probably precede all tooth extractions.

## PROGRAM

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SCIENTIFIC PROGRAM—OCTOBER 29, 1948

FRIDAY AFTERNOON, 2 00 P M

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### 13 RELATIONS BETWEEN VOLUMES OF CLOSED HYPOTHERMAL CEREBRAL LESIONS AND SYMPTOMS IN RABBITS

C BRUCE TAYLOR, M D (BY INVITATION), GEORGE M HASS, M D, AND  
JOHN E MALONEY, M D (BY INVITATION), CHICAGO, ILL

Acute closed cerebral lesions characterized by hemorrhage, necrosis, and progressive edema were produced hypothermally in rabbits without interrupting the continuity of the calvarium or introducing variables incidental to mechanical trauma. The dimensions and locations of lesions in the cerebrum were controlled so that they could be reproduced topographically and quantitatively in successive animals. Although hemorrhage, edema, and necrosis varied slightly in lesions which were otherwise identical, the variations were restricted to discrete volumes of injury.

When unilateral or bilateral lesions of the cerebrum occupied less than 9.4 volumes per cent of the brain, symptoms were negligible. When the lesions occupied 9.4 to 18.5 volumes per cent of the brain, severe symptoms developed in many animals. The data indicated that the minimum lethal volume of cerebral damage in 50 per cent of more than one hundred animals was 14.3 per cent of the volume of the brain. Severe clinical courses with an average postoperative duration of about seven hours and fatal termination always occurred when the lesions occupied more than 18.5 volumes per cent of the brain. A great majority of the animals that died had a normal postoperative period of behavior. Secondary lapse into stupor was a dependable indication of impending coma and eventual death within twenty-four hours after the time of completion of the operation.

These data indicate that a quantitative experimental approach to the problem of treatment of acute expanding closed intracerebral lesions characterized by local necrosis, hemorrhage, and edema can now be made. The postoperative duration of life can be predicted from the magnitude of the lesions which are produced and this duration is sufficient to permit evaluation of most therapeutic methods now used empirically in the treatment of acute expanding closed intracerebral lesions in human beings.

### 14 PARA-AMINOBENZOIC ACID THERAPY IN SCLERODERMA AND LYMPHOBLASTOMA CUTIS

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(INTRODUCED BY FRANK H BETHELL, M D)

Previous reports have dealt with the effects of para aminobenzoic acid (PABA) in leucemia, lupus erythematosus, dermatomyositis, and dermatitis herpetiformis. The purpose of this communication is to describe the results of PABA therapy in patients with scleroderma and with lymphoblastoma cutis.



The patients used in this study were made available by Dr. A. C. Curtis, Professor of Dermatology at the University of Michigan Medical School.

Five patients, presenting a wide range of sclerodermatous involvement, were treated with sodium (NaPAB) and/or potassium (KPAB) para-aminobenzoate. Improvement occurred in four and was most evident in the more extensively involved patients. The sclerodermatous areas gradually softened and became thinner with consequent increase in range of motion of the affected part. The fifth patient developed drug fever and therapy was discontinued.

Similarly, five patients with lymphoblastoma cutis were treated with NaPAB. All experienced relief from pruritus and objective improvement of the skin. This was characterized by diminution in erythema and infiltration. Treatment with NaPAB was discontinued in all five because of the development of edema. One of these patients was changed to KPAB therapy with loss of edema and striking improvement. Treatment was maintained for nine months in this subject. Of incidental interest has been the concomitant darkening during therapy of the patient's previously gray hair.

Administration of large amounts of PABA compounds results in glycosuria. This may not be verified in fermentation tests since the concentration of PABA in the urine of these patients will inhibit yeast fermentation activity.

All other forms of therapy for their respective disorders had been attempted in these patients before PABA therapy was undertaken. It would seem therefore, that PABA has a practical though limited value in the treatment of scleroderma and lymphoblastoma cutis. More important perhaps is the possibility that further study of PABA may yield information as to the mechanisms involved in these and related conditions of unknown etiology.

# 15 THE INHIBITORY EFFECT OF NITROGEN MUSTARD (BIS BETA CHLOROETHYL AMINE) ON THE DEVELOPMENT OF HUMORAL ANTIBODIES, CUTANEOUS HYPERSENSITIVENESS AND VASCULAR LESIONS IN RABBITS FOLLOWING INJECTIONS OF HORSE SERUM

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 KERRI S. WILSON, M.D. (BY INVITATION), AND MARY C. JOHNSON, A.B.  
 (BY INVITATION), ST. LOUIS, MO.

The vascular lesions that appear in the rabbit following the injections of horse serum are similar to those observed in certain diffuse vascular diseases in man. The latter are believed by some to result from hypersensitiveness. Because of these similarities the pathogenesis of the experimental lesions has been intensively investigated. The relationship of humoral antibody and cutaneous hypersensitiveness to the vascular lesions is still to be defined. Conflicting reports have been published concerning the influence upon the vascular lesions of various agents believed to affect antibody formation, antigen-antibody combination, or the cellular effects of the latter.

Antihistamines were used in our initial attempt to modify the vascular lesions. Neither Benadryl nor Neohetramine was observed to affect the lesions, the development of antibodies, or cutaneous hypersensitiveness.

Because of the ability of sulfur and nitrogen mustards to suppress antibody formation (Hektoen and Corpe, Phillips and co-workers and Spurr), we investigated the effect of bis beta-chloroethyl amine (BCE) on the formation of antibody as well as on the development of cutaneous hypersensitiveness and vascular lesions.

A group of rabbits weighing 20 to 25 kilograms were given, at four day intervals, a total of seven intravenous injections of 0.5 mg  $\text{HN}_2$  per kilogram. Two days after the third injection, each received 10 cc of horse serum per kilogram intravenously. A second group of rabbits received an identical dose of horse serum at the same time, but was not treated with  $\text{HN}_2$ . Two days after the seventh mustard injection and fourteen days after the serum injection, all animals were skin-tested with varying amounts of horse serum after which they were sacrificed. Blood serum samples obtained one week after injection of horse serum and again at the time of sacrifice were studied for the presence of horse serum antigen and antibody by precipitin tests. Pooled terminal serums of the two groups were separately analyzed quantitatively for total antibody nitrogen.

A marked leucopenia and moderate bleeding tendency developed in the  $\text{HN}_2$  treated animals but they otherwise appeared healthy and gained weight. Antigen persisted the same length of time in the untreated as in the treated rabbits. Antibody appeared after one week in the control animals but not until the end of the second week in the mustard-treated group. In the pooled terminal serums of the control group 0.062 mg antibody nitrogen per milliliter was present and but 0.031 mg in the treated. There was moderate suppression of skin hypersensitiveness in the  $\text{HN}_2$  treated group. Eighty per cent of the control group developed arterial and endocardial lesions whereas there were no vascular lesions observed in the treated animals.

While the data suggest that humoral antibody is important in the pathogenesis of the experimental vascular lesions, this has not yet been completely established, in view of the known effect of  $\text{HN}_2$  upon protein structures and enzyme systems other than those involved in antibody formation. In this regard we have found that  $\text{HN}_2$  in vitro had no effect upon the precipitation of rabbit antihorse serum by horse serum.

*Summary*—The nitrogen mustard, bis beta chloroethyl amine, was observed to suppress the development of humoral antibodies, cutaneous hypersensitiveness, and the vascular lesions in rabbits induced by horse serum.

## 16 EFFECT OF RUTIN UPON THE RESORPTIVE TOXICITY OF CERTAIN DRUGS

R. K. RICHARDS, M.D., NORTH CHICAGO, ILL.

Attempts to demonstrate an effect of rutin upon the capillaries by experimental methods have met with but limited success. Recently, reduction of trypan blue diffusion after local irritation of the skin of rabbits has been described (Ambrose and DeEds, *J. Pharm.* 90: 359, 1947), and also the prevention of radiation injury in rats (Griffith and co-workers, *Proc. Soc. Exptl. Biol. & Med.* 64: 332, 1947).

These procedures do not lend themselves readily to a quantitative measure of rutin action.

A new approach to this problem is based upon our early observation that sodium bisulfite in small concentrations (0.1 to 0.4 per cent) greatly increased toxicity of epinephrine or procaine if added to the drugs and injected intramuscularly or subcutaneously, but not intravenously. This "bisulfite phenomenon" was shown to be due to a local effect upon the capillaries by the bisulfite ion causing a faster absorption rate. Only drugs having a wide margin between the intravenous and the subcutaneous or intramuscular toxicity show a positive bisulfite phenomenon.

If rutin is injected intravenously in rats prior to the administration of the toxic drugs to which sodium bisulfite has been added the increased toxicity of epinephrine is greatly reduced without affecting the toxicity of epinephrine as such. This procedure permits a quantitative estimation of rutin action upon the capillaries.

A similar effect could be demonstrated against the increased toxicity of procaine caused by sodium bisulfite. However in contrast to the observations with epinephrine rutin also decreased toxicity of plain procaine solutions. It was shown that high concentrations of procaine exert a dilating effect upon capillaries which is antagonized by rutin.

Definite reduction of toxicity by previous rutin administration could also be shown with strychnine but little if any with Metrazol. The former of these two drugs has a much greater margin between the intravenous and the intramuscular toxicity than the latter thus the prolongation of the absorption produced by rutin permits the detoxification of the drug to become partially effective. The described procedures permit the use of bisulfite phenomenon and the decrease of the absorptive toxicity of certain drugs as a tool for further research on rutin on other drugs with specific effects upon the capillaries.

### EFFECT OF SEDATIVES ON URINARY VOLUME OF PREGNANT WOMEN

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(INTRODUCED BY W. M. FOWLER, M.D.)

One of the cardinal findings in toxemia of pregnancy is edema. In a previous communication before this Society we reported the effectiveness of various diuretic agents in the mobilization and excretion of sodium and water. This paper reports our observations on the antidiuretic effect of morphine and other sedative drugs in pregnant women.

A series of patients was given a constant intravenous infusion for five hours and hourly urine specimens were obtained for eight hours. On test days these patients were given injections of a sedative and the effect on the urinary output and chloride excretion was measured. Morphine reduced the urinary output by 50 per cent without any significant alteration in total urinary chlorides. The fluid retention was further manifested by a transitory increase in body weight.

Another series of patients was given oral fluids as a liquid breakfast of 1,000 cc during the hour of 7:00 to 8:00 AM. The urine volumes were then measured at hourly intervals for four hours. On test days the sedative was administered one hour after the liquid breakfast. Morphine, Demerol, codeine and Amytal caused similar depression in urinary volume. Paraldehyde and Verin have not been found to have an antidiuretic effect.

DeBodo reported that morphine caused a release of the antidiuretic hormone from the pituitary in dogs. Studies were undertaken to determine the mechanism of this antidiuresis in women. The similarity of response by normal subjects and patients with diabetes insipidus to morphine suggests that this antidiuretic effect is not mediated through the posterior pituitary. Since hypnosis did not induce this response it is apparent that the antidiuresis was effected by some mechanism other than sleep itself.

These observations suggest that morphine and similar drugs should be used with caution in edematous states. Tables and graphs will present these data.

## 18 EVALUATION OF NEUROGENIC AND HUMORAL FACTORS IN BLOOD PRESSURE MAINTENANCE IN NORMAL AND TOXEMIC PREGNANCY USING TETRA- ETHYLAMMONIUM CHLORIDE

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(BY INVITATION), AND EUGENE B. FERRIS, M.D.  
CINCINNATI, OHIO

Transient elimination of neurogenic tone by the action of sympathetic blocking agents has afforded a new physiologic approach to evaluation of mechanisms operative in sustaining blood pressure at normal or hypertensive levels. Tetraethylammonium chloride (TEAC), administered intravenously in 400 mg doses, induces a temporary blockade of impulse transmission at the autonomic ganglia. When neurogenic tone has been abolished in this manner, the arterioles remain responsive to humoral agents, and thus the remaining blood pressure (TEAC floor) must be maintained by humoral mechanisms together with intrinsic vascular tone.

Toxemia of pregnancy with its attendant hypertension has long been regarded as a humoral disorder despite the lack of physiologic evidence. In an effort to evaluate the relative importance of humoral and neurogenic factors in the blood pressure of patients with toxemia as well as those with normal pregnancy, we have utilized TEAC assay to study ten normal nonpregnant women, ten normal term pregnancies, eighteen cases of preeclampsia, and five cases of eclampsia.

In each of the normal term pregnancies, the prepartum response to autonomic block was a marked fall in blood pressure to mean levels of 55 to 65 mm Hg. Within twenty-four to forty-eight hours post partum, all these patients showed a striking rise in TEAC floors, the responses corresponding exactly to those of the normal nonpregnant controls.

Strikingly different responses occurred in the patients with toxemia. Pre partum TEAC floors invariably remained elevated above those of nonpregnant controls and were consistently 25 to 70 mm Hg higher than those of the normal pregnancies. With subsidence of toxemia post partum, floors promptly fell to the normal nonpregnant range. The diastolic TEAC floor appears to be of the greatest significance in these studies, for it never fell below 80 mm Hg in toxemia yet was never higher than 56 mm Hg in normal pregnancy. The highest diastolic floors consistently occurred in those patients who clinically were most toxic, regardless of the height of the pretest blood pressure.

The results suggest that (1) The hypertension of toxemia of pregnancy is supported primarily by an excessive degree of humoral tone. (2) Conversely, the blood pressure in normal term pregnancy is maintained largely by neurogenic and intrinsic tone. (3) Within forty-eight hours post partum of normal pregnancy there is a return to nonpregnant control mechanisms (humoral predominating). (4) Clinical assay with TEAC may be a helpful aid in diagnosis of toxemia of pregnancy and in the evaluation of changes in severity during its course.

## 19 THE EFFECT OF WATER BY VEIN ON THE MORE SEVERE COMPLICATIONS OF CARDIOVASCULAR RENAL DISEASE

F R SCHEIM, M D, AND J A LAYNE M D, GREAT FALLS MONT

Our most seriously ill patients showed the greatest need for plain water and were the least able to take or retain adequate amounts by mouth. With a proper regulation of sodium intake, including a minimal use of plasma, blood and other sodium laden solutions which yield no free water they were given plain water by vein in amounts designed to restore and maintain water balance.

The solutions used in about 500 periods of observation contained 50 or 25 Gm of dextrose (quickly oxidized or stored) in 1000 cc of distilled water which is quickly dispersed throughout the 50 liters of total body water. The usual volume given was 1,000 cc, with a range of from 500 to 2,000 cc at one time. The total amounts given in one day ranged from a 500 cc supplement to from 3,000 to 6,000 cc daily when the oral intake was nil or negligible. The rate of flow was from 10 to 20 cc per minute. (It would have required too many hours to administer the larger totals at slower rates and it was noted that reactions occurred more frequently with the fatigue and annoyance of long drawn out venoclyses.) The effect of the larger amounts of fluid by vein was observed over periods of from five to thirty five consecutive days.

There was no detectable clinical or laboratory evidence of harmful dilution observed immediately after a venoclysis or after several days of large amounts of these isotonic or hypotonic dextrose solutions. At the moment of completion of a venoclysis the changes in venous pressure were found to range from a drop of a few centimeters, through no change to a rise of as much as 13 cm. no objective or subjective evidence of left ventricular failure or pulmonary edema were noted with the rises in venous pressure. The venoclyses did not precipitate pulmonary edema in some cases which had recently experienced an acute profuse attack, and retinal edema and choked discs were seen to improve and convulsions came under control during some periods of observations.

In severe myocardial infarction with shock the water by vein was used effectively to prevent the development of anuria and azotemia, or for their correction. Severe congestive failure that had resisted sodium restriction and acid and mercurial diuretics was observed to respond to the increase in water intake made possible by intravenous supplements.

In many instances, what appeared to be an anuria with uremia due to so called renal shut down proved to be an anuria due to an inadequate intake of plain water. For water became available to the kidneys and they elaborated urine readily again, only after a large existing plain water deficit of from 4,000 to 7,000 cc and a high daily vaporization loss of 2,000 to 4,000 cc were provided for. In these patients the large amounts of plain water given sometimes entirely by vein did not induce heart failure and relieved the anuria and uremia without resort to decapsulation of peritoneal lavage.

## 20 THE NATURE OF THE ALTERED RENAL FUNCTION IN LOWER NEPHRON NEPHROSIS

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Serial determinations of clearances of mannitol, *p* aminohippurate, urea, and creatinine, and of tubular excretory mass were carried out in three subjects in the phase of diuresis and recovery from lower nephron nephrosis. In addition, urine-plasma ratios of nonprotein nitrogen were followed in these and three other patients with lower nephron nephrosis during the whole course of illness. With these data it was possible to extrapolate back to the period of oliguria and recognize the general nature of the functional alteration.

In two subjects the first function tests gave negative values for tubular excretory mass and impossibly high values for filtration fraction and ratio of *p*-aminohippurate clearance to tubular excretory mass. In subsequent tests these distortions disappeared, but the absolute values for clearances remained lower than normal. Complete restitution of renal function to normal did not occur until three to seven months had elapsed.

At the beginning of diuresis, the concentration ratio of nonprotein nitrogen remained as low as during the oliguric period. After several days of diuresis, the concentration ratio began to rise steadily even as the urine volume increased, indicating improvement in tubular function.

These data are consonant with the idea that the functional renal lesion in lower nephron nephrosis is a diminished renal blood flow in association with a loss of specific function of the lower nephron. Consequently, the limited amount of modified glomerular filtrate reaching the lower nephron is almost completely reabsorbed. A gradient diffusion through the damaged cells appears to be present which allows a greater reabsorption of *p*-aminohippurate than of mannitol and which is thus responsible for the distorted renal clearance tests. Recovery seems to be produced first by an increase in effective renal blood flow, followed later by a repair of tubular function. The gradient diffusion is apparently the first tubular defect to disappear. The total recovery of normal specific tubular function is much slower.

## 21 DANGERS FROM THE USE OF THE RICE DIET

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The present public interest in the Kempner rice diet led us to make a critical evaluation of this diet as an adjunct to the treatment of hypertensive heart disease. The results of this and more liberal diets have been recorded in the treatment of fifty-seven patients with hypertensive cardiovascular disease on the medical services of the Cook County Hospital and the Evanston Hospital. Supportive case reports point out that a simple convenient criterion of the urine specific gravity tends to indicate that one group of patients is benefited by the rice diet, another is unaffected, while still a third is made worse. The organism as a whole must be considered, nor can one overlook the fact that fundamental physiologic and biochemical principles govern the potential efficacy of this type of diet. One must be mindful of the physiology of extra and intracellular fluid, renal physiology, circulatory dynamics, and pulmonary ventilation of the role of blood, psychosomatics, and the endocrine system, and of the influence of medication. Blood protein and sodium levels must be given due considera-

tion. Illustrative groups of patients having hypertensive heart disease are presented, showing the influence of the renal reserve as reflected in the urine specific gravity and the influence of a variable extra renal environment. Twenty five patients with a good renal reserve indicated by a urine specific gravity of 1.020 or better were benefited by the rice diet (especially where cardiac decompensation was present). The improvement noted is attributable to the results of weight reduction by dietary restriction. Twenty four patients with a specific gravity range of 1.012 to 1.020 were possibly benefited by salt and protein restriction but again to a limited extent depending upon the status of the extra renal environment. Light patients with a specific gravity fixed at 1.010 were benefited at first but subsequently became worse when dietary restriction was continued. Serum sodium and chloride levels are altered, pre-renal azotemia with sequelae supervenes on a kidney with poor reserve and clinically the manifestations of weakness, lethargy, abdominal cramps, oliguria and disturbances in acid base balance become apparent. We consider urine specific gravity fixed at 1.010 as a warning signal to proceed with caution in the use of the rice diet and we believe it imperative to keep in mind the various possibilities which may be encountered especially in patients who have poor reserve in organs other than the kidney. When blood sodium levels begin to fall the rice diet should be discontinued. The narrow border between the pre-uremic state and uremia necessitates the addition of salt and blood or fluids to the therapeutic regime in order to reverse the process and thereby possibly avoid a casualty.

## 22 THE EFFECTS OF BACTERIAL PYROGENS ON MALIGNANT HYPERTENSIVE PERSONS

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(BY INVITATION), AND IRVING H PAGE, MD CLEVELAND, OHIO

Thirty five patients exhibiting the syndrome of malignant hypertension were treated with repeated injections of bacterial products which elicited leucocytosis and fever. The effects were evaluated from the levels of arterial pressure, cardiac, cerebral and renal functions and funduscopic changes. The responses to all the materials were so similar that they can be considered together.

The diastolic blood pressure of sixteen of the thirty five patients was reduced by treatment from 126 mm Hg mean (range 110 to 150) to average 100 mm Hg during five to nineteen weeks. Papilledema disappeared in all but two, one of whom still is receiving treatment. The persistent elevation of the optic disc in the other may represent a lesion of the nerve. Fourteen patients became free of retinal hemorrhages. One of the remaining two is still under treatment and, in the other, the severity grading was reduced from 4 to 1. Most of the fresh exudates disappeared however in nine persons scars of previous disease persisted.

Before treatment, five of these sixteen patients presented signs of congestive heart failure, all evidences of which were relieved during therapy. Electrocardiograms showing signs of damage became normal in seven and improved in the other nine. Heart size (teleroentgenogram) diminished from plus 18 per cent before to plus 5 per cent after treatment.

Renal function at first was moderately depressed in nine patients but was eventually restored to or above control levels.

Except for persistent elevation of arterial pressure remissions have lasted for an average of twenty four months and up to thirty months.

Eleven of the sixteen patients who responded well are living after fourteen to thirty one months. Three of the remaining five discontinued treatment after

three to thirteen weeks and died within three months of cerebral hemorrhage. Another died of apoplexy ten months after treatment with pyrogen and six months after sympathectomy. The fifth, the only one with return of the malignant phase, died of cerebral hemorrhage six months after treatment was begun.

Of the remaining nineteen patients, eleven responded more briefly and in less degree than the sixteen described, while eight showed no change. All but two are dead. These two are at present under treatment.

Tolerance to pyrogens appeared in five to nineteen weeks, after which arterial pressure rose, but usually without reappearance of the malignant syndrome.

The outcome was not predictable from arterial pressure, the retinopathy, or the extent of cardiac disease. Six patients who were virtually blind and four who had congestive failure responded favorably. The significance of renal function is shown by the fact that TmPAH averaged 62 mg per 1.73 sq M in the sixteen who responded well (range 48 to 109), the mean was 26 (range, 12 to 40) in those responding poorly, and 20.8 (range, 7 to 32) in those with no response.

In summary, the malignant phase of essential hypertension can be remitted in some patients by repeated injections of bacterial pyrogens provided renal damage has not advanced to the extent that TmPAH is less than 45 per milligram per minute per 1.73 sq M of body surface.

### 23 A TEST FOR THE PRESENCE OF THE HYPERTENSIVE Diencephalic Syndrome Using Histamine

HENRY A. SCHROEDER, M.D., AND MELVIN L. GOLDMAN, M.D. (By Invitation)  
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A syndrome associated with arterial hypertension has been described by Page which he called the hypertensive diencephalic syndrome. Occurring usually in women, but also in men, it is characterized by labile hypertension and attacks of a periodic appearance of a blotchy rashlike blush on the face, chest and shoulders, associated often with headache, lacrimation, and signs of vasoconstriction in the extremities. Attacks of this disorder probably represent some neurogenic disturbance associated with hypertension. In susceptible individuals this syndrome has been reproduced by the intradermal injection of 0.25 mg of histamine, reaching its height five to ten minutes after the injection. Of fifty patients with normal blood pressure, the syndrome was made to appear in five. Of thirty-three with neurogenic hypertension it appeared in twenty-six. In fourteen patients with other types of arterial hypertension it appeared in none. Four subjects in whom the reaction to histamine was marked failed to react after lumbodorsal sympathectomy. When the reaction occurred, the symptoms of which the patient complained usually were reproduced, including the typical hypertensive headache. Antihistaminic agents only partially blocked the reaction in one subject. It was not reproduced by the administration of epinephrine or Mecholyl. The possible role of histamine like substances in causing some of the symptoms of neurogenic hypertension is suggested.



## PROGRAM

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SCIENTIFIC PROGRAM—OCTOBER 30 1948

SATURDAY MORNING, 9 15 A M

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### 24. GLYCOSURIA SIMULATED BY THE ADMINISTRATION OF ASCORBIC ACID ITS OCCURRENCE AND DIFFERENTIATION

JOHN C MITHOEFER, M D (BY INVITATION), AND CARL I WILTER M D  
CINCINNATI, OHIO

The confusion afforded by repeatedly positive Benedict tests on urine of a diabetic patient whose diabetes was thought to be well controlled and who was receiving large doses of ascorbic acid prompted us to investigate the reducing power of this substance in the urine as measured by Benedict's qualitative test and the Clinitest tablet.

Quantitative *in vitro* experiments were performed in order to determine the amount of ascorbic acid which must be present in solution to give a positive Benedict test and Clinitest. It was found that 0.2 mg of ascorbic acid was sufficient to reduce 5 ml of Benedict solution to a 1 plus reaction and the Clinitest to a 'trace'. 1.9 mg of ascorbic acid produced a 2 plus with both tests. 2.1 mg, a 3 plus while 8.0 mg were required to give a 4 plus reaction. It was found that when compared by weight ascorbic acid was a more powerful reducing substance than glucose when tested by these methods. It was further shown that ascorbic acid and glucose when present together in solution have an additive effect in this reduction reaction.

*In vivo* experiments showed that when ascorbic acid was administered in large doses parenterally (now a common surgical practice) or by mouth there was usually sufficiently rapid urinary excretion of this substance to give a positive Benedict test or Clinitest. Following the intravenous administration of 500 mg of ascorbic acid the majority of the patients gave a 1 plus Benedict test in two hours and had a urinary output ranging between 38 and 136 mg per cent ascorbic acid. Following the intravenous administration of 1,000 mg of ascorbic acid, all but one of the patients had a 2 plus Benedict test after two hours. The urinary concentration of ascorbic acid ranged from 430 to 980 mg per cent at that time.

A simple method depending on oxidation of ascorbic acid in alkaline medium has been devised for differentiating this substance from glucose in the urine. Two milliliters of urine are made alkaline with sodium carbonate followed by the addition of 6 to 8 drops of hydrogen peroxide. This procedure rapidly destroys the reducing power of ascorbic acid but does not interfere with the reducing power of glucose as measured by Benedict's test and the Clinitest. This procedure is useful not only in the differentiation between glycosuria and a false positive Benedict test due to ascorbic acid in routine urinalyses but also enables one to follow more accurately the glycosuria of a diabetic patient who is receiving large doses of ascorbic acid.

## 25 "STEROID DIABETES" ASSOCIATED WITH CUSHING'S SYNDROME AND EXCRETION OF 17-HYDROXYCORTICOSTERONE (COMPOUND F) IN URINE, METABOLIC STUDIES

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ROCHESTER, MINN

The diabetes which is commonly associated with Cushing's syndrome is probably explainable on the basis of an overproduction of carbohydrate active steroid hormones by hyperplastic or neoplastic adrenal cortices. Such diabetes should, therefore, resemble the "steroid diabetes" produced by Ingle and co-workers in rats by the administration of 17-hydroxy-11 dehydrocorticosterone (Compound E) or 17-hydroxycorticosterone (Compound F), that is, it should differ from ordinary diabetes, particularly of the juvenile type, in being more resistant to insulin and in being mild during fasting. Furthermore, since one of the effects of the carbohydrate-active steroids is to stimulate the production of sugar from protein precursors, this type of diabetes should be characterized by the excretion of relatively large amounts of nitrogen in the urine, even when glycosuria is minimized by the use of diet or insulin, or both.

Observations which lend support to the foregoing ideas were made in the case of a 14-year-old boy with severe diabetes associated with Cushing's syndrome due to hyperplastic adrenal cortices. In addition to diabetes, the patient presented most of the classic features of Cushing's syndrome, including hypertension, osteoporosis, muscular weakness, and a hypochloremic, hypokalemic alkalosis of marked degree. That the diabetes was due to an overproduction of carbohydrate-active adrenal steroids was suggested by the fact that the urinary excretion of "cortinlike substances" ("11-oxysteroids") was remarkably high (about 17 mg daily, or about 200 times normal). Indeed, 17 hydroxycorticosterone was isolated from the urine, 191 mg of purified hormone being obtained from a twenty-five day collection of urine.\*

Metabolic studies revealed that the diabetes exhibited the features of "steroid diabetes" in animals. It was of more than usual severity (in terms of insulin requirement), glycosuria being incompletely controlled with 130 units of insulin given daily while the patient received 187 Gm of carbohydrate daily in the diet. Unlike the usual behavior in ordinary juvenile diabetes, however, the urine became virtually free of sugar (24 Gm in twenty-four hours) and ketonuria was absent when food and insulin were withheld for twenty-four hours. (This amelioration of glycosuria with fasting and withholding of insulin was in marked contrast to the behavior of patients having both Addison's disease and diabetes who were previously studied by us, in whom withdrawal of insulin and fasting during the administration of Compound E resulted in the rapid development of severe diabetic acidosis.) As in the case of steroid diabetes in animals, nitrogen balance was decidedly negative with an intake of 57 Gm protein daily. Likewise, as suggested by the presence of osteoporosis, the balances for calcium and phosphorus were also negative.

The patient died after surgical resection of one hyperplastic adrenal gland. At necropsy pronounced hypertrophy and hyperplasia of the adrenal cortices, a small thymoma, a parathyroid adenoma (not hyperfunctioning), osteoporosis and bilateral renal calculi were found.

The clinical behavior of the diabetes, the metabolic data, and the finding of large amounts of 17-hydroxycorticosterone (Compound F) in the urine seem

\*Mason H L and Sprague R G Isolation of 17-hydroxycorticosterone From the Urine in a Case of Cushing's Syndrome Associated With Severe Diabetes Mellitus J Biol Chem 175 451-456 1948

to justify the conclusion that the patient had 'steroid diabetes' analogous to that produced in animals by the administration of carbohydrate active adrenal steroids

## 26 ROLE OF THE ADRENAL CORTEX IN URATE METABOLISM AND IN GOUT

WILLIAM D ROBINSON, M D JEROME W CONN, M D WALTER D BLOCK, PH D  
(BY INVITATION) AND LAWRENCE H LOUIS, SC D (BY INVITATION)  
ANN ARBOR, MICH

Urate metabolism on a constant diet has been studied in three normal and one gouty subject during administration of approximately 50 mg (Armour's Standard) of purified pituitary adrenocorticotrophic hormone intramuscularly daily, in divided doses for five to ten consecutive days. By using a specific enzymatic method employing uricase to determine blood and urine urates errors, inherent in the conventional colorimetric methods were avoided.

Following the administration of adrenocorticotrophic hormone to normal subjects, the urate excretion increased from 50 to over 100 per cent above base line values. This increase appeared during the first twenty four hours and was well sustained reaching its peak on the sixth seventh and ninth injection days respectively, in the three subjects. It was accompanied by an increase in excretion of nonurate chromogen in all three but this was irregular and not sustained during the injection period. There was an apparent decrease in blood urate when determined by direct colorimetric methods which are known to be influenced by other reducing substances but no significant change in the blood content of urate or of nonurate chromogen as measured by the uricase method. On the first postinjection day there was a sharp drop in urinary urate excretion to near basal levels, followed by a definite increase on the second to fifth post injection days. There was associated other evidences of a rebound in pituitary adrenal cortical activity at this time.

The injection of desoxycorticosterone acetate in doses of 20 mg daily to a normal subject for ten days did not produce any significant effect on urine or blood urate levels.

The chief differences in response of the gouty patient to adrenocorticotrophic hormone as contrasted to normal subjects were (1) absence of a significant increase in excretion of nonurate chromogen (2) a sharp decrease in true blood urate to less than one half of the base line level so that loss from body fluids by simple increased renal excretion could account for one half to three fourths of urinary increase as compared to normal subjects where only negligible quantities could be accounted for on this basis and (3) absence of any secondary increase in urate excretion during the postinjection period. Effects on blood sugar, glucose tolerance glycosuria urinary nitrogen amino acid and 17 ketosteroid excretion were similar to those seen in the normal subjects, except for the absence of any evidence of a rebound in hormonal activity during the postinjection period.

The gouty subject who had been free of attacks for nine months preceding study had a mild but definite attack on the third to fifth postinjection days at a time when all data suggested a depression of adrenal cortical activity. Talbott and associates (1935-1940) described cyclic changes in urate electrolyte, and water excretion in patients with gout and related these changes to acute gouty attacks. Their data can be interpreted as reflecting cyclic changes in adrenal cortical activity with attacks occurring during periods of decreased

function. A common denominator for many of the incidents which have long been recognized as capable of provoking acute attacks of gout is furnished by their ability to affect pituitary-adrenocortical activity.

## 27 INFLUENCE OF PHYSICAL FITNESS AND CONVALESCENCE FROM MAJOR SURGICAL PROCEDURES ON THE MAGNITUDE OF THE RESPONSES TO EXERCISE OF THE BLOOD AND URINE LACTATE

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MAURICE GEPHARDT, M.D. (BY INVITATION), JOSEPHINE M. DYNIEWICZ,  
P.H.C. (BY INVITATION), SOPHIE J. PRESLEY, M.D. (BY INVITATION),  
AND GEORGE LAVERS, M.D. (BY INVITATION)  
CHICAGO, ILL.

The changes in the values of the blood and urine lactate produced by exercise on a treadmill were studied in a group of fifty patients, in various states of physical fitness, before and after hemorrhaphy or cholecystectomy.

Prioperatively, with the same exercise load (three minutes on the treadmill), the maximal lactic acid level attained in the blood and the total quantity in the postexercise urine specimen were found to be much greater in patients in poor physical condition than in those in excellent shape. Generally, similar differences were noted in the case of submaximal and maximal exercises, despite the fact that the respective periods on the treadmill for individuals in good physical condition were three to six times as long as for those in poor shape.

Postoperatively, one group of nineteen patients was placed at complete bed rest for from six to thirteen days. Except for some individuals in excellent physical condition, the patients showed much higher maximal blood lactate levels with exercise during convalescence than was observed preoperatively. Very rarely were the readings back to the preoperative level by the twentieth postoperative day. The changes in the quantity of lactate in the postexercise urine specimens followed the same trend in some instances the values remaining elevated for as long as sixty days after operation. It is of interest that the lactate studies continued to show abnormal changes postoperatively long after the results of the tests of liver function and of circulatory and respiratory efficiency had returned to preoperative levels.

Other groups of patients subjected to the same operative loads were placed on regimens involving early ambulation. Except for occasional patients initially in poor physical condition, these individuals showed, during convalescence, smaller increases in maximal blood lactate levels and lactate content of the urine with exercise, as compared with the preoperative readings, than were observed for the patients subjected to a period of postoperative bed rest.

It is concluded that the magnitude of the blood and urine lactate changes produced by exercise is definitely influenced by the state of physical fitness of the individual. Furthermore, lactate studies are of value in the measurement of the length of convalescence following surgical operations and in the evaluation of various therapeutic regimens used to reduce this period.

## 28 PORTAL CIRRHOSIS OF THE LIVER A CLINICAL AND FUNCTIONAL STUDY

WILLIAM L. RICKFITS, M.D. (BY INVITATION) JOSEPH B. KIRSNER, M.D.,  
AND WALTER L. PALMER, M.D., CHICAGO, ILL.

Fifty cases of portal cirrhosis of the liver were studied by biopsy and tests of hepatic function. The fifty cases were divided into three groups.

Group I included fourteen patients free of symptoms, except one who had hematemesis and melena, but with physical findings suggesting the presence of cirrhosis such as hepatomegaly, splenomegaly or collateral circulation of the abdomen. In two, the cirrhosis was first noted at laparotomy. The presence of cirrhosis was established by histologic examination. The alterations in hepatic function were minimal or absent, the bromsulfalein test was the most consistently abnormal.

Group II included twenty three patients in whom signs and symptoms of moderate degrees of hepatic disease were present. Fibrosis and hepatocellular changes were noted histologically in all. Function tests in this group were uniformly abnormal. Repeated studies of hepatic function were made in nineteen of the group during the course of medical management. There was no significant change in two, improvement was marked in fifteen, and the test ultimately yielded normal results in two.

Group III included thirteen patients with marked jaundice and other symptoms of hepatic dysfunction requiring hospitalization. The function tests were markedly impaired in all. Fibrosis and severe parenchymal changes were noted histologically in ten of the thirteen. Four were admitted desperately ill and died soon after hospitalization. Subsidence of symptoms, marked improvement in hepatic function and regeneration of the liver parenchyma were observed accompanying medical management in nine. The symptoms disappeared completely in six of these, two improved greatly and one has improved and has no symptoms other than an acquired hemolytic anemia.

The following impressions were gained: (1) Abnormalities of hepatic function in portal cirrhosis depend upon the alterations of the parenchyma. (2) Fibrosis of the liver per se does not change the tests of liver function. (3) Portal cirrhosis may be present without detectable abnormality of hepatic function, and (4) portal cirrhosis is not necessarily a progressive disease.

## 29 SERIAL SERUM CHOLINESTERASE DETERMINATIONS IN LIVER DISEASE

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CHICAGO, ILL.

(INTRODUCED BY ROBERT W. KEETON, M.D.)

It has long been known that the cholinesterase activity of the serum is abnormally low in patients with disease of the liver and its determination has been suggested in the past as an index of liver function. However, because of the difficulty in performing determinations of serum cholinesterase activity, and because of the wide range of normal values obtained such determinations have not become popular. The recent development by Michel of a simple potentiometric method for the determination of cholinesterase activity now makes

this measurement a practical clinical tool. The method measures the change in pH due to the liberation of acetic acid in the hydrolysis of acetylcholine by cholinesterase.

To date serum cholinesterase activity has been estimated in forty-two patients and serial determinations have been made in twenty-six of these patients with liver disease, as well as in four normal subjects. In patients with liver disease serial determinations have correlated well with changes in clinical status at times when a battery of currently accepted liver function tests failed to show significant changes.

Serial determinations are reported of the serum cholinesterase activity of seven patients with cirrhosis of the liver and three healthy controls living in a metabolic ward for periods of 70 to 240 days. Determinations were made three times weekly before, during, and after intravenous therapy with human serum albumin containing less than 0.3 Gm. of sodium in each 25 Gm. of albumin. The total doses of albumin ranged from 125 Gm. in a three-day period to 3,600 Gm. in three periods totaling forty-seven days. It was found that six of the seven patients with liver disease showed a significant rise in the serum cholinesterase activity together with subjective and objective clinical improvement. In all of these cases the serum cholinesterase activity began to rise toward normal levels before improvement was detectable by any other laboratory means employed. Results suggest that serial determinations of cholinesterase activity afford a more sensitive index to slight changes in hepatic function than any of the other tests used in this study (that is, cephalin flocculation, thymol turbidity and flocculation, BSP excretion, serum bilirubin, immediate and total, two-hour Watson urinary urobilinogen, prothrombin time, total proteins with A/G ratio).

Studies are in progress on the rate of regeneration of serum cholinesterase activity following its destruction by di-isopropyl fluorophosphate. In thirteen patients with liver disease, as well as in four healthy controls, the return to serum cholinesterase activity to pretest levels has taken approximately four weeks, the healthy controls starting at and returning to a higher value than the patients.

### 30 ANTIDIURETIC ACTIVITY OF URINE IN ACUTE HEPATITIS

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(INTRODUCED BY O. O. MEYER, M.D.)

This study was undertaken in an attempt to confirm the presence of an antidiuretic factor in the urine of patients with liver disease and to elucidate the mechanism of its appearance.

Daily twenty-four hour urine specimens were obtained from nine patients with acute infectious hepatitis, one with serum hepatitis, and one with an acute febrile and icteric exacerbation of a portal cirrhosis with ascites and edema.

Protein precipitation at pH 4.0 and 80° C. was carried out, the supernatant was dialyzed twenty-four hours against tap water and evaporated to 100 ml. in cellophane sacks before an electric fan.

Assay was by intraperitoneal injection of 0.5 ml. per 100 Gm. body weight into groups of four adult male albino rats hydrated to 5 per cent of their body weight by tap water gavage. Controls received tap water or similar extracts from normal urines intraperitoneally. Urinary output per 100 Gm. of body weight was plotted for three- or five-hour periods and compared with the intake output records and clinical appearance of the patients.

In the early phases of acute hepatitis there was a sharp inhibition of diuresis in the assay animals which paralleled clinical evidences of a tendency to water retention. With beginning convalescence this inhibitory activity lessened and the output of assay animals approached or exceeded the controls. In two instances the first specimen obtained from a patient failed to show antidiuretic activity, although subsequent ones during the early phase of illness did. Similar results were seen over a longer period in the encephalic patient.

Samples of varying activity were assayed on male albino rats made encephalic by prolonged twice weekly injections of carbon tetrachloride. The degree of inhibition of diuresis in this group did not exceed that in simultaneously run normal control animals, and indeed, in about two thirds was slightly less.

Five and eight hour urine outputs from both the control and encephalic animals were extracted in similar fashion being evaporated to 12 ml and re-assayed. These preparations showed no increase in antidiuretic activity as compared with similar recoveries from animals injected with tap water.

These studies confirm Labby's findings of the presence of a factor showing antidiuretic activity in the urine of patients in the early phases of acute hepatitis. They do not support the thesis that failure of hepatic inactivation of a normally formed principle of this nature is the basis of its presence.

### 31 VITAMIN B<sub>1</sub> IN PERNICIOUS ANEMIA AND PUERPERAL MACROCYTIC ANEMIA

FRANK H BETHELL MD MURIEL C MYERS MD (BY INVITATION) AND  
ROSALIE B NELIGH, MD (BY INVITATION) ANN ARBOR MICH

Four patients with pernicious anemia in relapse were treated with vitamin B<sub>1</sub>\* for periods of ten to twenty weeks. Intramuscular injections of 1 µg of B<sub>1</sub> were given daily during the period of the reticulocyte response with subsequent treatments at varying intervals up to twenty five days. Doses were adjusted so that the equivalent of 1 µg per day would be received. Three of the patients had maximum reticulocyte levels and rates of red cell increase which exceeded the expected response following daily injections of 1 U S P unit of liver extract. The fourth patient received 1 mg of the folic acid antagonist, Aminopterin† daily for two days prior to B<sub>1</sub> administration and for the first fourteen days of treatment with B<sub>1</sub>. The reticulocyte response was delayed and suboptimal. When aminopterin was discontinued a second reticulocyte response occurred with rapid elevation of red cell count. All four patients attained normal hematologic values after six to eight weeks.

Three patients had nervous system involvement. Following B<sub>1</sub> therapy there was relief of paresthesias and decrease in ataxia. Glossitis was troublesome in three patients and disappeared promptly on institution of treatment.

A girl aged 19 years was seen two months after delivery of a normal infant. The presenting features were severe anemia, intense glossitis, stomatitis and vulvovaginitis. For at least two years she had received an extremely poor diet. Red cell count was 1,500,000 with macrocytosis, leucopenia and megaloblastic marrow. Gastric contents contained hydrochloric acid. Intramuscular injections of 1 µg of B<sub>1</sub> for ten days failed to produce hemopoietic or clinical responses. Anemia and leucopenia became more severe and mucous membrane lesions progressed. Megaloblastic marrow reaction persisted. Subsequent treatment with folic acid 10 mg daily by mouth was followed by conversion of

\*Merck & Co. Inc.  
†Lederle Laboratories American Cyanamid Co.

marrow picture, reticulocyte response, increase in erythrocyte and leucocyte values, and rapid subsidence of stomatitis and vulvovaginitis

Assays of feces of four patients with untreated pernicious anemia revealed high contents of growth-stimulating factor for *Lactobacillus lactis* Doinei, the test organism for vitamin B<sub>12</sub>\*. The content of B<sub>12</sub> equivalent ranged from 3 to 18  $\mu$ g per gram of dried feces. Thus the daily output of B<sub>12</sub> by patients with pernicious anemia appears to be many times greater than that necessary to produce remission when introduced parenterally. This suggests that in pernicious anemia there may be defective absorption of vitamin B<sub>12</sub> derived either from dietary sources or by intestinal bacterial synthesis.

## 32 STUDIES ON GLOBIN AND PORPHYRIN METABOLISM MADE WITH C<sup>14</sup> AND N<sup>15</sup>

MOISES GRINSTEIN, PH D (BY INVITATION), M D KAMEN, PH D (BY INVITATION), AND CARL V MOORE, M D, ST LOUIS, MO

Three different forms of tracer-tagged glycine have been used to label hemoglobin. Shemin and Rittenberg employed glycine containing N<sup>15</sup> to demonstrate that this amino acid serves as a nitrogenous precursor of protoporphyrin, a small amount of the N<sup>15</sup> was also incorporated in the total cell protein. They followed the N<sup>15</sup> content of heme carefully and found that (a) the labeled protoporphyrin within red cells does not participate in the dynamic metabolic state, (b) is not re-utilized for heme formation when red cells are destroyed, and (c) serves as a reliable index of the length of life of the erythrocyte. Altman and associates, using methylene labeled glycine (C<sup>14</sup>), have shown that the alpha carbon of glycine is utilized for the biosynthesis of hemoglobin protoporphyrin; a relatively small amount of C<sup>14</sup> is incorporated into globin. When glycine labeled with C<sup>14</sup> in the carboxyl position is fed to animals, the C<sup>14</sup> is built into globin but not into protoporphyrin (Grinstein and co-workers). The first and last of these three methods of tagging hemoglobin were used in the experiments described below to study globin and porphyrin metabolism.

Glycine containing C<sup>14</sup> in the carboxyl group was fed to two dogs and to one rat. In each instance, the C<sup>14</sup> appeared in the globin portion of hemoglobin but could not be demonstrated in the heme. The concentration in globin rose rapidly in the dogs during the first few days, remained relatively constant until the sixty-fifth and eightieth days, respectively, and then fell to very low levels. The shape of the curve obtained was very similar to that found by Shemin and Rittenberg for heme labeled with N<sup>15</sup>. The results indicate that the globin of intact erythrocytes (1) remains within the cell during its life span without participating in the dynamic protein interchange characteristic of nucleated cells and (2) is apparently not utilized again for new hemoglobin formation once the cell is destroyed.

In the second series of experiments, 5.6 Gm of glycine tagged with 27.7 per cent excess N<sup>15</sup> was fed to one dog which had previously been made anemic by bleeding. From blood obtained at intervals during the following thirty-four days, crystalline protoporphyrin 9 dimethylester was isolated and found to be tagged with the isotope (0.72-0.96 atom per cent excess N<sup>15</sup>). During the first twenty days of the experiment, coproporphyrin I (tetramethylester) was isolated from the urine and feces of this animal, it contained N<sup>15</sup> in a relatively high concentration (1.1 atoms per cent excess). On the thirty-fourth day, the dog was bled to death, and the total volume of red blood cells removed was trans-

\*Performed by Dr O D Bird Parke Davis & Co



fused into a second anemic dog. The following day, crystalline protoporphyrin 9 isolated from the blood of the recipient animal contained 0.40 atom per cent excess  $N^{15}$ . An acute anemia was then induced with phenylhydrazine. Coproporphyrin I isolated from the combined urine and feces during this period had no demonstrable  $N^{15}$  (less than 0.03 atom per cent excess), while the protoporphyrin 9 dimethyl ester isolated at the same time from urine and feces did contain the isotope (0.133 atom per cent excess). On the twentieth day when the animal was recovering from the phenylhydrazine anemia protoporphyrin 9 dimethyl ester crystallized from its blood had  $N^{15}$  in a concentration of 0.132 atoms per cent excess. This experiment has not yet been repeated. Conclusions therefore must be regarded as being *preliminary*. The data suggest that coproporphyrin I is formed as a by product during the biosynthesis of hemoglobin and is not derived from hemoglobin catabolism. This result was expected. More surprising, however, was the increased concentration of  $N^{15}$  in the protoporphyrin 9 from the excreta of the recipient dog, a finding which suggests that the excreted protoporphyrin 9 might, in phenylhydrazine anemia at least be a derivative of hemoglobin degraded during the hemolysis. Free erythrocyte protoporphyrin of the red cells would be another possible source of the fecal protoporphyrin 9.

### 33 THE COAGULATION DEFECT IN THROMBOCYTOPENIC PURPURA

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In thrombocytopenic purpura the platelets are reduced, the bleeding time is prolonged, clot retraction is absent, spontaneous hemorrhages occur, and petechiae and other manifestations of vascular fragility appear. The coagulation time characteristically is nearly always normal. This has led to the tacit acceptance that no true coagulation disturbance is present in this disease and that platelets are of secondary importance in the coagulation mechanism.

Recent findings concerning the platelets makes it possible to offer a new concept of coagulation and thereby to formulate a better explanation for the basic hemostatic dysfunction in thrombocytopenic purpura. It is now certain that platelets are indispensable for coagulation. When these cells disintegrate they liberate an enzyme which immediately acts on the precursor of thromboplastin (thromboplastinogen), converting it to its active form. This product acts on the prothrombin complex to form thrombin. When platelets are diminished, the conversion of thromboplastinogen is delayed and therefore the activation of prothrombin is abnormally slow. This is easily measured by the prothrombin consumption test which furnishes a better estimate of the true coagulability than the coagulation time since the latter is merely an empirical measurement of the time required for the formation of enough fibrin to meet an artificially set standard such as sufficient solidity to hold the blood when the test tube is inverted.

The prothrombin consumption is markedly reduced in severe thrombocytopenic purpura. The coagulation time may however even be shorter than normal and therefore mask the basic coagulation disturbance. In hemophilia the prothrombin consumption likewise is very low. Thus, the basic defect in adequate formation of thrombin is common to both hemophilia and thrombocytopenic purpura. Yet the clinical manifestations of these two diseases are distinctly different. One finds moreover occasionally a persistent low platelet count without a concomitant spontaneous hemorrhagic diathesis and a prolonged bleeding time. It is likely therefore that the characteristic features of

thrombocytopenic purpura such as the petechiae, the mucous membrane oozing of blood, and the prolonged bleeding time are not due directly to the deficiency of platelets but are caused by a factor affecting the vascular structure, particularly the capillaries, and this is superimposed upon the basic coagulation defect. In summary, the poor prothrombin consumption and the defective clot retraction are due directly to lack of platelets, while the spontaneous hemorrhagic tendency is attributable to a vascular factor whose action is accentuated by the coagulation defect.

### 34 A STUDY OF THE PLASMA DEFECT IN PATIENTS WHOSE BLEEDING IS TEMPORARILY CONTROLLED BY PROTAMINE SULFATE OR TOLUIDINE BLUE

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BURTON J GROSSMAN, B S (BY INVITATION)  
CHICAGO, ILL

The anticoagulant action of heparin is rapidly overcome upon addition of protamine (salmine sulfate). Normally when a standard amount of heparin is added to a constant volume of blood (0.09 mg of liquid heparin per milliliter of blood), its action is regularly overcome and clotting occurs with a standard amount of protamine sulfate, 0.120 mg. In certain hemorrhagic states, most but not all of which are associated with thrombocytopenia, considerably more protamine may be required before clotting occurs. These patients generally have a prolonged Lee-White clotting time, the clotting of which is preceded by a gel-like formation. This syndrome has been observed in patients with leukemia who are bleeding, patients under extensive nitrogen mustard and/or P<sup>32</sup> therapy, a small percentage of patients with idiopathic thrombocytopenic purpura, in three patients who received heavy "spray" x-radiation, in some patients who bleed abnormally but in whom the platelet count and other known clotting studies are normal, and in certain patients with menorrhagia.

The nature of the defect is not known. It may be due to the release of heparin or heparin-like substances since the defect measured in this heparin-protamine titration procedure may be temporarily corrected by the administration of protamine sulfate or toluidine blue. The possibility of a decrease of heparin co-factor associated with a heparinemia (?) has been explored. This possibility was suggested by the fact that the amount of heparin required to alter the heparin-protamine titration in normal patients or dogs renders the Lee-White clotting time incoagulable. This is in contrast to the defect seen in the patient with this bleeding syndrome whose clotting time may be only moderately prolonged but whose heparin-protamine titration is remarkably altered. However, since *in vivo* rapid intravenous heparin (0.25 to 0.50 mg per kilogram) administration in such patients or in irradiated dogs has a much greater effect upon the whole blood clotting time than usually occurs, it appears unlikely that the heparin co-factor is abnormal.

Since many of the plasma and tissue proteins are antiheparins, it may be that the associated protein changes in some of these disorders in part account for the disturbance through the release of heparin or heparin-like substances, which may then affect the clotting system. Present studies suggest that a diminution in the antiheparins of plasma and/or an increase, relative or actual, of the plasma heparinoid substances are the systems most likely affected. More data are required before a final conclusion can be drawn.

### 3. CHANGES IN BLOOD AND BONE MARROW IN ACUTE LEUCEMIA INDUCED BY AMINOPTERIN

J M STICKNEY, M D   A B HAGEDORN, M D   S D MILLS, M D, AND  
TALBERT COOPER, M D  
ROCHESTER, MINN

(INTRODUCED BY F J HECK, M D)

4 Aminopteroyl glutamic acid (Aminopterin) was administered to eight  
teen adults and children with acute leukemia diagnosed by study of the blood  
and bone marrow. Remissions in the clinical course of the disease as well as  
improvement in the blood and bone marrow were observed in some instances,  
although many patients made no response to the drug. Toxic manifestations  
included stomatitis, diarrhea, alopecia, deafness and temporary aplasia of the  
bone marrow. In an attempt to prevent these toxic effects vitamin B complex,  
crude liver, and folic acid were used.

When the response to treatment was favorable changes in all the elements  
of bone marrow were conspicuous. The myeloid cells assumed a more normal  
pattern and in lymphatic leukemia the lymphocytes decreased and became more  
mature. The increase in erythropoiesis was striking. When folic acid or liver  
extract was not administered the marrow sometimes contained megaloblasts  
and hyperlobulated polymorphonuclear leucocytes. Liver or folic acid pre-  
vented megaloblastic changes or caused them to disappear after they were estab-  
lished.

These observations are further confirmation of the theory that Aminopterin  
has an action antagonistic to that of folic acid. The length of time over which  
the clinical observations have been made does not permit any definite conclu-  
sions about the eventual effect of Aminopterin in acute leukemia.

### ADDITIONAL ABSTRACTS

#### 36 THE MINIMAL SODIUM DIET A CONTROLLED STUDY OF ITS EFFECT UPON THE BLOOD PRESSURE OF AMBULATORY HYPERTENSIVE SUBJECTS

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The administration of a diet rigidly restricted in sodium may influence the habits and reactions of the patient profoundly, altering blood pressure through mechanisms unrelated to the effect of sodium restriction per se. A controlled evaluation of the factors attendant upon the use of this diet is necessary, particularly in the nonhospitalized patient. Accordingly, twenty-one hypertensive subjects were placed on a calorically adequate diet estimated to contain less than 300 mg of sodium per day and were followed for approximately eighteen weeks. Blood pressures were obtained in the clinic by a constant technique, and twenty-four hour urine collections were made once a week. All subjects received medication, consisting either of 4 Gm of NaCl a day or of identical appearing placebos. After each six-week period the medication was either changed or continued according to prearranged schedules, initially selected at random for each subject by the pharmacist.

The experiment thus consisted of three periods, differing only in that during one (or two) period(s) supplemental sodium chloride was administered, while in two (or one) period(s) the sodium intake remained at a bare minimum. The subject, whose cooperation was solicited, was unaware of the nature of the medication, and during the study the investigators did not know which medication was being taken.

*Results*—Data from which evaluation of the effect of rigid sodium restriction upon blood pressure could be based were available from only eight of the subjects. The criteria for their selection required that the twenty-four hour urinary sodium average below 500 mg for at least one period and over 1,000 mg for at least one period. For these subjects the average of the blood pressures taken during rigid sodium restriction was lower than the average during the periods of added sodium, by 4.72 mm Hg diastolic (range, 18.5 to -8.02) and 4.96 mm Hg systolic (range, 22.15 to -7.90).

All twenty-one subjects yielded data for at least two periods of a like order of sodium excretion, from which the variances of the blood pressures for the group were determined. On this basis, the probability that the experimentally observed differences could be due to chance is less than 1 in 50 for the diastolic values ( $t = 3.01$ ) and less than 1 in 20 for the systolic values ( $t = 2.36$ ).

#### *Conclusions*—

- 1 A diet rigidly restricted in sodium is difficult to administer successfully to ambulatory hypertensive subjects.
- 2 A difference of less than 5 mm Hg in average blood pressure was observed, ascribable to the effect of NaCl restriction alone.
- 3 This difference is statistically significant.

# 37 THE EFFECT OF DESOXYCORTICOSTERONE ACETATE AND PROPYLENE GLYCOL IN EXPERIMENTAL RENAL HYPERTENSION

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WILLIAM JACOBS, M D (BY INVITATION) NEW ORLEANS, LA

Experiments performed to determine the blood pressure response in renal hypertensive and normal dogs revealed the following (1) a small but definite pressor effect from intravenous administration of propylene glycol in unanesthetized dogs (2) no additional effect from intravenous administration of desoxycorticosterone acetate dissolved in propylene glycol (3) no pressor effect from intravenous administration of desoxycorticosterone glucoside and (4) a similar and sustained pressor effect from continued subcutaneous administration of desoxycorticosterone acetate in sesame oil in both normal and hypertensive dogs

Initial experiments were performed on mongrel dogs trained for blood pressure determinations by femoral arterial puncture. Three were made hypertensive by renal arterial clamps or perirenal silk wrappings. Approximately eighteen months were allowed as the postoperative stabilization period. Desoxycorticosterone acetate in daily doses of 0.15 to 1.28 mg per kilogram of body weight was given for a total of eight periods of six weeks each in three hypertensive and two normal dogs. Blood pressure rises of 25 to 50 mm Hg were observed in seven instances. These were sustained and disappeared within two to four weeks of withdrawal of the drug. Repeat courses of the drug in the same animals produced shorter and less pronounced elevations of blood pressure which tended to return to control level before the drug was discontinued. In some instances the post treatment levels were 20 to 30 mm Hg lower than control levels. There were no essential differences between the responses of the hypertensive and the normal dogs.

In a second group of experiments propylene glycol and desoxycorticosterone acetate in propylene glycol were given intravenously to three hypertensive and three normal dogs. In twenty six determinations no significant difference between normal and hypertensive blood pressure responses to the two preparations was noted. Two cubic centimeters of propylene glycol regularly produced a rise of 15-30 mm Hg in both groups. The response was unchanged by the addition of 5 mg of desoxycorticosterone acetate.

Aqueous desoxycorticosterone glucoside in dosage of 5 mg intravenously had no pressor effect in any instance. In an acutely hypertensive dog anesthetized with pentobarbital sodium no significant pressor effects were observed with any of the three preparations.

# 38 INTRA-ARTERIAL BLOOD PRESSURE CURVES DURING TILTING

## A NEW METHOD OF STUDYING HYPERTENSION

ROBERT S GREEN M D (BY INVITATION) ARNOLD IGLEAUER M D AND  
JOHNSON MCGUIRE M D CINCINNATI OHIO

Previous studies demonstrated that the blood pressure at the level of the aortic arch invariably rose during a tilt from 20 degrees erect to a 45 degree head down position and fell during the return tilt to the 20 degree erect position. These alterations were due to a change of the effect of the force of gravity on the central blood column.

These changes apparently acted as intra-arterial stimuli to blood pressure regulatory mechanisms. In normal subjects, maintained in the altered position, the primary elevation that occurred during the head down tilt was followed by a secondary reflex depressor response, the fall during the erect tilt by a pressor response, whereby the blood pressure returned within eight to eighteen seconds to approximately the initial level, regardless of position.

Similar studies have been completed in a series of fifty nonselected patients with hypertension. Primary mechanical alterations of blood pressure occurred during tilting as in the normal group. However, the secondary response to these alterations varied.

The elevation during the head down tilt was followed by four types of response:

- 1 Delayed depressor the blood pressure rose further before falling
- 2 Decreased depressor the blood pressure remained at an elevated level for a prolonged period
- 3 Normal depressor
- 4 Increased depressor the pressure shortly after the head down tilt was lower than in the semierect position. Patients with this type response usually complained of dizziness on bending over.

The fall during the erect tilt was also followed by four types of response:

- 1 Increased pressor the blood pressure immediately surged beyond, then gradually returned to the initial level of the 20 degree erect position
- 2 Normal pressor
- 3 Decreased pressor the blood pressure remained lower than the initial erect level for a prolonged period
- 4 Delayed pressor the blood pressure fell further before returning to the initial erect level

Patients with the two latter types of response usually complained of dizziness on arising suddenly.

Various combinations of pressor and depressor response occurred in individual patients, that is, normal depressor with increased pressor, decreased pressor with normal pressor, etc. In a given patient the combination of response remained constant on successive tiltings.

In normal subjects the heart rate invariably slowed following the head down tilt. In some hypertensive patients the heart rate slowed as in the normal, in others it did not change.

It is hoped that this method of study of the cardiovascular reflexes will offer a means of classifying hypertension and indicate possible approaches to treatment.

### 39 ANTIBODIES AGAINST RENIN AND "SUSTAINED PRESSOR PRINCIPLE" PRODUCED BY INJECTIONS OF KIDNEY EXTRACTS

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Intramuscular or intraperitoneal injection of various kidney extracts into several animal species caused the appearance in the sera of a substance or substances capable of destroying or "neutralizing" renin and the "sustained pressor principle."

Extracts prepared from hog kidneys were administered over the period of a year to a series of ten hypertensive patients. Although antibodies against

hog renin and hog 'sustained pressor principle' were formed in the serum of these patients, the antibodies failed to neutralize human renin or 'sustained pressor principle'. These extracts which did not possess irritating and shock producing properties caused no lowering of blood pressure in the patients.

Of the several types of animals used only the dog produced antibodies to its own renin when injected with heterologous kidney extracts.

#### 40 EFFECTS OF SPLANCHNICECTOMY ON BLOOD PRESSURE AND ON CARDIAC AND RENAL FUNCTION

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ANN ARBOR MICH

One hundred eight hypertensive patients were studied before and ten to sixteen months after supradiaphragmatic splanchnicectomy (Peet). Thirty one control patients acceptable for the operation who did not undergo surgery were re-examined ten to eighteen months later under similar conditions. These patients were comparable to the operated group in age, sex, height of blood pressure and duration of hypertension and were treated by the usual medical methods including mild sedation in some cases. Table I presents the significant differences in the observations on the two groups at the time of follow up examination.

TABLE I

INITIAL STATUS OF PATIENT	POST SPLANCHNICECTOMY SERIES		CONTROL SERIES	
	NUMBER	% IMPROVED	NUMBER	% IMPROVED
Casual diastolic blood pressure exceeding 110 mm mercury	80	21*	25	0
Cardiac enlargement present by teleroentgenogram	15	26	5	0
Electrocardiographic abnormality present	81	22	9	0
Headaches	84	92	20	50
Irritability and nervousness	73	72	16	25
Dizziness	50	76	13	54

\*More than 20 mm decline in casual diastolic blood pressure at follow up visit was considered improved.

Only twelve of the patients had a diastolic blood pressure below 90 after operation. We believe, however, that there were definite evidences in certain of the other hypertensive patients that the operation improved their clinical status when they were compared with the control subjects. This result was accomplished with a minimum postoperative disability. Patients left the hospital ten to twelve days after operation and were able to resume full activity in two to three months.

Sixteen of the splanchnicectomized patients who had had a 20 mm decline in diastolic blood pressure were compared with seventeen patients who had experienced slight increases in pressure one year after operation. In general the subjects in the group which improved were younger, had a higher initial blood pressure, contained more females and showed a greater fall in blood pressure after tetraethylammonium than did those in the group showing poor results.

In thirty four patients renal hemodynamics were studied before and at various time intervals after operation. Renal blood flow (para aminohippurate clearance) was usually unchanged but filtration rate (mannitol or thiosulfate

clearance, tended to fall with a consequent decline in filtration fraction. In some patients with a 20 per cent postoperative reduction in mean blood pressure the renal blood flow was maintained and there was a consequent decline in renal resistance suggesting some postoperative renal vasodilatation.

#### 41 THE ARTERIOLES OF THE SKIN IN ESSENTIAL HYPERTENSION

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It is generally accepted that a diffuse disturbance of the arterial side of the vascular system exists in hypertension. Curiously enough the cutaneous arteriolar beds in people with essential hypertension have not been studied adequately. For this reason we felt that a controlled study of the arteriolar bed of the skin in a group of persons who had essential hypertension might be of value.

Material for this study was obtained from the upper arm lumbar region and calf or flit-two persons who had normal blood pressure and from seventy persons who had moderate to severe essential hypertension. The skin was obtained by means of excision and punch. All the material in the hypertensive group was secured from living people. The youngest person with hypertension was 29 years old, the oldest person was 80 years of age, the mean age was 44 years. Of the flit-two specimens of skin removed from persons who had normal blood pressure forty were taken from living people and twelve were obtained at necropsy.

Sections for microscopic study were made. A Bausch and Lomb micrometer eyepiece was used over a high-power objective which produced a magnification of 430 times (43 x 10). With this instrument the average thickness of the wall of the vessel and diameter of the lumen were measured. We studied every slide from left to right and made measurements of the first four arterioles we saw in each slide.

A measurable thickening of the arteriolar wall and a decrease in the wall-to-lumen ratio as compared with normal were found in vessels of the skin of patients with hypertension. The average of the wall-to-lumen ratio of the arterioles of fifty-two persons with normal blood pressure was 1.214, among seventy persons who had essential hypertension it was 1.157. Not all arterioles were equally affected in the same case.

Qualitative changes were also present. Hyperplasia of the nuclear elements of the media and thickening of the inner elastic lamina appeared to be the most common changes. Occasionally, complete occlusion of the lumen occurred. Although the arterioles in the skin of persons who had malignant hypertension were more profoundly altered than were those of persons who had other types of hypertension, equally severe qualitative changes were present in the arterioles of a number of persons who had the other types of hypertension. Four patients with normal blood pressure had medial hypertrophy of the arteriolar wall.

#### 42 RETINAL ARTERIOVENOUS NICKING

II. A LONG-TERM STUDY OF THE DEVELOPMENT OF ARTERIOVENOUS NICKING IN PATIENTS WITH HYPERTENSION

SAMUEL A. SHELBURNE, M.D., DALLAS, TEXAS

We reported our studies on the significance of various stages of retinal arteriovenous nicking in patients with hypertension before this society in November, 1939. We have now re-studied a group of these same patients after eight



to ten year intervals and have followed for the first time the development of arteriovenous nicking from the earliest stages to the latest. We have thus shown for the first time that the so called early lesion actually develops into the late lesion. The importance of distinguishing these lesions one from the other will be emphasized. Color drawings of unusual clarity will be presented showing the various types of arteriovenous nicking and other retinal arterial changes found in hypertensive patients.

### 43 THE INSULIN TOLLRANCE OF THE HYPERTENSIVE PATIENT

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CHICAGO, ILL.

The insulin tolerance of fifty hypertensive patients have been studied. One tenth of a unit of insulin per kilogram of body weight was injected intravenously and blood sugar levels were determined at 15, 30, 60, 90 and 120 minutes after each injection. A normal response was characterized by a drop of the blood sugar to about 50 per cent of the fasting level at thirty minutes followed by a return to normal in the next two hours.

In the present series of fifty patients 16 per cent showed complete and 8 per cent showed a partial resistance to the intravenous dose of insulin. This insulin response was used to measure the presence of corticoadrenal activity. Such hyperactivity may be one of the causes of immediate failure following splanchnic nerve section although delayed improvement may occur.

In addition, our previous observations that the insulin resistant diabetic patient becomes insulin sensitive after splanchnic nerve section was observed in two patients with diabetes in this series.

### 44 PALPITATION

EDWARD MASSIE, M.D., AND HERBERT C. WIGAND, M.D. (BY INVITATION)  
ST. LOUIS, MO.

Palpitation is a symptom usually much more alarming than serious. It consists of an unpleasant sensation of the heart's action whether slow or fast, regular or irregular. It more frequently does not indicate primary physical but rather a psychic disturbance.

Four hundred patients from Barnes Hospital, private practice and the Washington University Clinics presenting various degrees of palpitation were studied to determine the clinical characteristics of this condition. Of this group approximately half were clinic and the other half hospital patients. Sixty four per cent were females and 36 per cent males. Palpitation was noted as a primary complaint in approximately half the entire group and as an accessory complaint in the remainder. The commonest age incidence was in the group from 40 to 50 years and next in order of frequency were the sixth and fourth decades. One hundred ninety five (49 per cent) had a severe form of palpitation, 183 (46 per cent) had this symptom to a moderate degree and 22 (5 per cent) considered it mild. In women the complaint appeared more consistently in the severe form.

Of the 152 patients with cardiac and vascular disease the principal etiologic factors included arteriosclerosis and hypertension (53 per cent), hypertension alone (18 per cent) and rheumatic fever (7 per cent). Seventeen per cent of the total group had extracardiac conditions of diverse type. The arrhythmias encountered in the 325 patients who had electrocardiograms in

cluded ventricular extrasystoles (22 per cent), auricular fibrillation (12 per cent), sinus tachycardia (9 per cent), sinus bradycardia (6 per cent), and very few instances of auricular extrasystoles, auricular flutter, and paroxysmal auricular tachycardia. Two patients had complete heart block. Two hundred twenty-seven patients had basal metabolism tests, only three had rates above +30 per cent, and nine were in the range from +20 to +30 per cent. Only six patients had clinical hyperthyroidism.

Two hundred twenty-eight (57 per cent) of the entire group presented primary nervous complaints, and of these patients, 57 per cent had a definite anxiety state, 22 per cent were typical of neurocirculatory asthenia, 12 per cent presented prominent menopausal symptoms, and 9 per cent were psychotic. Analysis of the cases revealed no significant evidence that such factors as anemia, fever, digitalis, or smoking had any direct connection with this symptom. Excessive intake of alcohol or coffee seemed to produce marked palpitation. Undoubtedly the most common etiologic factor was the presence of a significant anxiety state, and when this was associated with emotional stress or fatigue, palpitation became not only a frequent, but also a most troublesome symptom.

#### 45 OBSERVATIONS ON ONE HUNDRED PATIENTS WITH MYOCARDIAL INFARCTION WHO SURVIVED UP TO SIX YEARS

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We have recently had the opportunity to make a follow up survey on 100 patients who had survived an acute myocardial infarction for from one to six years.

Our patients represented a cross section of the population with varied occupations, interests, and income. Most of the patients had returned to their former jobs. In most instances the younger patients were more regularly able to resume a full share of previous activity after recovery than the older ones.

In general the trend was toward a higher blood pressure at follow up than that which existed at the time of infarction. Males were more prone to have hypertension with the greatest incidence on the fifth decade. Hypertension was the most frequent finding among patients with little electrocardiographic restitution.

Angina pectoris occurred four times as frequently in males as in females, with the greatest incidence in the sixth decade. There appeared to be no relationship between angina pectoris and the amount of electrocardiographic restitution.

Heart failure was seen most frequently in patients in the sixth decade. Almost all of these patients showed little electrocardiographic restitution.

Combinations of angina, hypertension, and heart failure were most frequent among males than females, especially in the fifth and sixth decades. None of the patients with complete electrocardiographic restitution had a combination of angina, failure, or hypertension.

More than five times as many males as females were "asymptomatic" the greatest incidence was in the fourth decade. Patients with previous lateral or atypical infarction electrocardiographic patterns were most likely to be asymptomatic. Approximately two-thirds of the asymptomatic patients had no disturbances of conduction, rhythm, or voltage at the time of their infarction.

46 INTRAVENOUS CATHETERIZATION OF THE HEART IN THE  
DIAGNOSIS OF CONGENITAL HEART DISEASEDON W CHAMMAN, M.D., LLOYD J GUGLE M.D (BY INVITATION)  
HOUSTON, TEXAS

Recent advances in surgical alteration or correction of certain congenital defects of the heart and great vessels make it imperative that more accurate diagnosis of such lesions be made. Intravenous catheterization of the heart has been a useful aid to ascertain the condition in patients suspected of having such congenital abnormalities.

A 6 French or 9 French special catheter is introduced into the median basilic vein and passed under fluoroscopic control via the subclavian vein and superior vena cava into the right side of the heart and into the pulmonary artery and its branches. The oxygen content of samples of blood are obtained at various sites and should not differ normally by more than 1-9 volumes per cent. Pressures in the various sites are recorded by means of a Hamilton manometer.

This procedure has been used in 58 patients with or suspected of having congenital defects of the heart or great vessels. The results illustrate its value to ascertain whether or not the patient has a defect to indicate the operability when defects are discovered and to suggest the prognosis.

In cases of auricular septal defect the oxygen content of the blood from the right atrium is greater than that in the vena cavae. In patients with ventricular septal defects, a significant increase in oxygen content in the blood from the right ventricle is demonstrated when compared with that in the atrium. Septal defects may also be demonstrated by passing the catheter through the defect into the left side of the heart. Patent ductus arteriosus is diagnosed by finding an increase in oxygen content of blood from the pulmonary artery as compared with the right ventricle and occasionally by an increased pulmonary arterial pressure. Cases of cyanotic congenital heart disease are presented showing the usual absence of left to right shunt. Cyanotic patients with increased pressure in the right ventricle or pulmonary artery are discussed. Combined lesions such as cyanotic congenital heart disease with patent ductus arteriosus may be found and patients with such abnormalities are described. Several patients who were suspected of congenital defects but in whom the catheterization studies were normal are described. A case in which a pulmonary vein was found to empty into the right (?) or common (?) atrium is reported. Complications of the procedure including premature ventricular contractions, phlebitis and venospasm, are discussed.

47 DIETARY AND HORMONAL INFLUENCES IN EXPERIMENTAL  
ANURIAG MASSON, PH.D (BY INVITATION) A C CORCORAN, M.D., AND  
IRVINE H PAGE, M.D. CLEVELAND OHIO

The importance of diet in the treatment of uremia has been recently emphasized by Borst. The present study was devised to test experimentally the effects of high iso caloric (9 Cals per 100 sq cm body surface per day) carbohydrate fat and protein diets and of the provision of hormones (desoxy corticosterone acetate and free testosterone 5 mg daily of aqueous suspension) on survival times and azotemia (blood urea N) in bilaterally nephrectomized rats. All treatment was begun at the time of nephrectomy.

Data are summarized in Table I. Survival was definitely improved and azotemia greatly diminished by administration of fat or carbohydrate rather than protein. Survival was somewhat more prolonged and azotemia decreased by administration of carbohydrate rather than fat.

TABLE I MEAN SURVIVAL TIMES AND BLOOD UREA NITROGEN LEVELS IN BILATERALLY NEPHRECTOMIZED RATS

DIET	TREATMENT	NUMBER OF ANIMALS	SURVIVAL TIME (HR.)	BLOOD UREA NITROGEN (MG)	
				AT 30 HR.	AT 48 HR.*
Protein	0	40	42	264	550
	DCA	10	44	263	543
	Testosterone	10	44	240	461
	DCA and testosterone	10	52	281	510
Carbohydrate	0	20	93	112	134
	DCA	9	127	89	131
	Testosterone	9	118	100	130
	DCA and testosterone	9	116	60	90
Fat	0	10	74	135	175
	DCA	9	79	113	167
	Testosterone	9	74	121	173
	DCA and testosterone	9	71	122	166

\*In Survivors

Treatment with hormones had no significant effect on survival time regardless of diet, and little effect on azotemia. The data indicate a decrease in blood urea in rats treated with DCA and testosterone on the high carbohydrate diet. It is not established that this change is significant and it is, in any case, evident that it does not improve survival.

These experiments substantiate the beneficial effects claimed by Borst for the treatment of uremia by administration of a high caloric diet, composed largely of carbohydrate. They do not confirm the suggestions of others that hormonal treatment is of value after nephrectomy.

#### 48 PLASMA PROTEIN STUDIES IN CHILDREN WITH RHEUMATIC FEVER

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Recent statistical studies completed by this clinic revealed an unexpectedly low recurrence rate for a highly susceptible group of young rheumatic children who had received special attention to improve their diets and level of home care. Prior to management, the diets of these children commonly were found to be deficient in protein. The relation between analysis and course of the disease was studied. In general the electrophoretic partition between the albumin and the total globulin fractions confirmed the chemical analysis. The fibrinogen fraction tended to be higher by the electrophoretic method. During the more active phase of rheumatic fever the values for plasma albumin were consistently and markedly decreased, while the values for gamma globulin, alpha globulin and fibrinogen were increased. The changes in the gamma globulin fraction were particularly pronounced. With improvement in the clinical condition of the child the distribution of the plasma proteins shifted toward the normal. Return of the sedimentation rate and the plasma albumin to normal values generally occurred simultaneously, but exceptions were observed.

## 49 THE RELIEF OF EDEMA IN NEPHROSIS BY PLASMA ALBUMIN AND GELATIN INFUSIONS

WARREN B COOKSEA, M D, DETROIT MICH

Seven patients with advanced nephrotic edema who had been treated by the usual methods without benefit, were given large amounts of salt poor plasma albumin intravenously. There were two failures one of which was a death from infection. Four cases progressed to a satisfactory remission after the marked diuresis which was obtained and one case is still pending. Three patients have been diuresed most satisfactorily with 10 per cent gelatin in distilled water and three have been treated with both gelatin and albumin with very satisfactory results. These experiences strongly suggest that a greatly hastened remission in cases of the nephrotic syndrome is often possible because of the effective diuresis brought about by this method.

## 50 THE ACTION OF SEVERAL CARDIAC GLYCOSIDES ON EXCITABILITY AND CONDUCTION VELOCITY IN THE DOG HEART

GORDON K MOE M D, AND RAFAEL MENDEZ M D (BY INVITATION)  
MEXICO D I

Digitoxin (Digitaline Nativelle), lanatoside C (leditamid) ouabain (Ainaud), and katiophanthoside (Strophosid) were studied in dogs under chloralose anesthesia. The heart was exposed and electrodes attached at various points on the surface of the right auricle and both ventricles to permit measurement of A V conduction linear conduction on the surface of the ventricles in response to electrical shocks and the threshold of electrical excitability of both auricle and ventricle. The glycosides were injected at thirty minute intervals in doses calculated to kill the heart in about four hours.

Auricular excitability began to diminish after the first dose, reaching 50 per cent of normal at 55 to 70 per cent of the lethal dose. Auricular arrest occurred at about 80 per cent of the lethal dose when auricular excitability was from 17 to 45 per cent of normal. Shortly thereafter the auricles became completely inexcitable.

Ventricular excitability increased slightly (10 to 20 per cent) after the first dose, but after the administration of 60 to 80 per cent of the lethal dose began to diminish rapidly reaching levels of 50 to 60 per cent of normal shortly before death. Although the first idioventricular beats were recorded at a time when the ventricle was more than normally excitable the frequency of extra systoles increased leading eventually to multifocal ventricular rhythms and fibrillation, as the excitability diminished below normal levels. The ectopic activity produced by toxic doses of digitalis cannot therefore be explained in terms of electrical excitability but rather of increased automatism.

A V conduction, as expected was slowed progressively after the first dose until complete block occurred at 55 to 70 per cent of the lethal dose. Severe impairment of A V conduction was evident before any slowing of intraventricular conduction could be measured. Ventricular conduction from a stimulating to a recording electrode less than 15 mm distant was rarely delayed and often accelerated even in the terminal stage of intoxication. The time interval between near and distant electrodes was not altered until about 70 per cent of the lethal dose had been administered but then increased progressively until death. Since propagation of a forced beat from the point of stimulation to a near electrode must represent chiefly muscular conduction and to a distant

point chiefly Purkinje conduction, it would appear that digitalis specifically depresses the specialized conducting tissue. Digitalis fibrillation becomes possible when conduction in the Purkinje tissue has been depressed so much that an excitation wave has not yet reached the most distant fibers at the time when a new impulse has begun.

## 51 ETIOLOGY OF AURICULAR FIBRILLATION AND THE MECHANISM OF ITS PERPETUATION

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(INTRODUCED BY L. N. KATZ, M.D.)

Observations on man and the dog are presented which relate to the etiology of auricular fibrillation and the mechanism of its perpetuation. Vagal stimulation and anoxia are the main etiologic factors in the initiation and perpetuation of auricular fibrillation.

Vagal stimulation (mechanical and chemical) may induce auricular fibrillation. Acetylcholine injected directly into the blood stream was used in our experiments to produce chemical vagal stimulation. Moderate anoxia reduces the threshold of the auricles to the initiation of fibrillation but does not induce this arrhythmia per se, marked anoxia, on the other hand, increases the threshold to initiating fibrillation.

Anoxia of the auricles was found or produced (1) by interference with or obstruction of its vascular supply, (2) by a decrease in the amount of oxygen carriers, (3) by a decreased oxygen content of the blood due to anoxic anoxemia, and (4) by interference with tissue respiration. The relationship between vagal stimulation and anoxia can be plotted in a graph and on this correlation the cause of the perpetuation of auricular fibrillation can be demonstrated. The clinical implication of these findings and the therapeutic approach to this problem are illustrated and discussed.

## 52 DICUMAROL AND QUINIDINE IN THE AMBULATORY TREATMENT OF CHRONIC AURICULAR FIBRILLATION

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This report consists of a study on thirty-three cases of chronic auricular fibrillation treated from the outpatient department of the Los Angeles County General Hospital.

The purpose of this investigation was to (1) add preventive measures against possible thrombus formation in the auricle and thereby help eliminate the probability of embolic phenomena after restoring a chronic fibrillating heart to normal rhythm, (2) to re-establish normal sinus rhythm, (3) To re-evaluate the difference between the cardiac output in a slow fibrillating heart and that of the heart after it has been restored to normal sinus rhythm.

### *Material —*

*Sex* Twenty-nine males, four females

*Age range* 17 to 83 years

*Etiology* Rheumatoid group, twenty-one cases, arteriosclerotic and hypertension group, twelve cases

*Duration of fibrillation* Approximately two years

*Technique*—(1) Patients were first digitalized (2) Circulation time venous pressure, and vital capacity were then determined (3) Dicumarol was administered until a prothrombin level of between 30 and 20 per cent was obtained (4) Quinidine therapy was then given in daily increasing doses The maximum amount of quinidine given any patient in a twenty four hour period was 30 grains (5) After normal sinus rhythm was obtained circulation time, venous pressure, and vital capacity were again determined

*Results*—In eighteen of the thirty three patients 55 per cent normal rhythm was restored It was also restored in ten patients 48 per cent of the rheumatoid group and eight 67 per cent of the arteriosclerotic and hypertension group

Laboratory findings in the restored group *Vital capacity* increased on the average of 20 per cent *Venous pressure* lowered on the average of 60 mm *Circulation time* either increased on the average of 3 seconds Calcium gluconate increased on the average of 8 seconds

### 53 EXPERIMENTAL STUDIES ON THE COMBINED ACTION OF DIGITALIS (CEDILANID) AND QUINIDINE (IN CATS)

S A WEISMAN, M D, LOS ANGELES CALIF

The meaning of the term 'combined action' of quinidine and digitalis on the heart, as discussed in the literature perhaps warrants some clarification It usually refers to the action of these drugs on the heart after they have been given following each other at varying time intervals

The purpose of this study was to (1) reinvestigate the pharmacologic action of digitalis and quinidine on the heart after giving these drugs in consecutive order at varying time intervals (2) to study the effect of these drugs on the heart after both drugs are administered together and (3) to reinvestigate the effect of quinidine on respiration following the administration of digitalis and the effect on respiration after these drugs are given simultaneously

Comparative studies were made on the (1) mortality (2) heart rate, (3) blood pressure, (4) electrocardiographic changes (5) respiration

*Results*—It was found that there appeared to be less toxic effects on the heart and the respiration after these drugs were administered together than when they were given in consecutive order

The clinical application of these observations is discussed

### 54 PAROXYSMAL TACHYCARDIA LOW NODAL IN ORIGIN BENEFITED BY CARDIAC SYMPATHECTOMY

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WESLEY A GUSTAFSON, M D (BY INVITATION) CHICAGO ILL

A 35 year old, cyanotic, white female a complete invalid because of incapacitating paroxysms of tachycardia lasting up to fifteen minutes in duration and occurring thirty to forty times per day was admitted to the hospital for study

General medical history was of cyanosis since birth rheumatic fever at 6 years of age diphtheria at 7 mastoidectomy at 7 at 22 years she had had a cerebrovascular accident with hemiplegia and aphasia which essentially cleared in six months

The exact nature of the congenital cardiac anomaly was not determined, but it was clinically diagnosed as cor triloculare biatriatum. The laboratory work-up revealed a slight polycythemia (hematocrit, 55 per cent, RBC, 5.5 million) and electrocardiographic evidence that the origin of the impulse of the cardiac stimulus was either low nodal or high in the bundle of His.

The first attack of paroxysmal tachycardia noted by the mother was when the patient was 3½ years of age, and the attacks had progressed from once a month to the present frequency. It was early noted that vomiting caused a cessation of the fast rate and thus and other manipulations were utilized by the patient. Electrocardiographic tracings were taken when the patient would initiate a paroxysm of tachycardia by exertion and then stop the same by pharyngeal stimulation. Cardiac asystole up to 3 seconds in duration followed this conversion of rhythm.

It was surprising that the autonomic nervous system exercised control over cardiac impulse of such low origin. To establish the presence of this control a pharmacologic approach was taken, yielding the following results. Atropine sulfate, given by hypodermic needle, within ten minutes produced a tachycardia which persisted for several hours, quinine sulfate in minimal doses resulted in an increase in frequency and duration of the paroxysms of tachycardia, Prostigmine produced no change, digitoxin in therapeutic and near toxic doses gave a decrease in frequency and duration of the paroxysms, diisopropyl fluorophosphate resulted in decreased frequency and duration of the paroxysms (Di-isopropyl-fluorophosphate destroys cholinesterase which in turn allows enhancement of the vagus through prolonged action of acetylcholine).

In the light of these findings it was felt warranted to remove the cardiac autonomic accelerators (the cardiac sympathetics) in an attempt to stabilize the cardiac rhythm at its slower rate. A two stage bilateral cardiac sympathectomy of stellate ganglion and the first, second, third, fourth, and fifth thoracic nerves was done.

Since surgery the patient has experienced only about five paroxysms of tachycardia per day, lasting only a few seconds.

## 55 THE EFFECT OF APPREHENSION CAUSED BY THE TECHNICAL PROCEDURES ON CARDIAC OUTPUT

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In the course of studies of the effect of spinal anesthesia on cardiac output, the variability due to apprehension was so extreme as to make the results of these studies wholly unreliable because initial control values within normal basal range could seldom be obtained. Furthermore the cardiac output varied unpredictably during the course of the procedures. The variations from standard and normal values were always high.

This report is based on a study of cardiac output by the direct Fick method in twenty-four individuals. There were never less than three serial determinations of cardiac output during an experimental period in any patient. In nine patients four consecutive cardiac outputs were done at each experimental period.

It was our belief that high values were due to apprehension. In general, the rate of oxygen consumption bore out this belief. Attempts to use the appearance of the patient, pulse rates, and respiratory rates as criteria for excitement failed to correlate with either our cardiac output results or with the oxygen consumption.



When control values of cardiac output were within normal limits, spinal anesthesia to the first thoracic nerve did not cause decrease in cardiac output. When control cardiac output values were high spinal anesthesia caused them to drop to normal levels. This is probably due to temporary denervation of the heart caused by the high spinal anesthesia.

Our frequent inability to obtain normal cardiac output values for control observations caused us to sedate our patients with Nembutal morphine, and scopolamine prior to and during the observations. The uniformly normal control cardiac outputs which we obtained in twelve patients with such sedation have been so satisfactory as to make us believe that sedation is essential in studies which are directed at determining the effect of certain procedures on cardiac output.

## 56 THE EFFECTS OF ACUTE HYPOXIA ON THE SENSITIVITY OF THE HEART TO ACETYLCHOLINE

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S ROBBARD MD (BY INVITATION) AND L A KATZ MD CHICAGO, ILL

This laboratory has recently been engaged in a study of the physiologic responses of the circulation to acute hypoxia. We have shown that during acute severe hypoxia the response of the blood vessels to epinephrine is greatly reduced and that this substance persists in the blood beyond the hypoxic period when relief of hypoxia is quickly introduced. The effect of hypoxia on the action of acetylcholine on the cardiac pacemakers has also been analyzed. In the present study the effect of hypoxia on the sensitivity of the heart to acetylcholine was measured.

In nine dogs catheters were inserted into the left femoral artery and passed under fluoroscopic control into the region of the sinuses of Valsalva. One hundred two injections of acetylcholine were used during hypoxia and the decrease in rhythmicity and conductivity which was produced was recorded electrocardiographically. The threshold dose of acetylcholine injected via the catheter required to produce slowing or block was established during air breathing before and after each hypoxia test. Nitrogen breathing was substituted for air to produce the hypoxia. It was found that the sensitivity of the heart to acetylcholine increased progressively with the continuation of nitrogen breathing, as illustrated in Table I.

TABLE I

DURATION OF HYPOXIA (SEC)	INCREASE IN R-R DISTANCE (SEC)	PRESENCE OF A-V BLOCK
Control	0	0
30	0.3	0
60	0.5	+
100	1.1	+
140	1.7	+
180	2.5	+
220	3.8	+

Relief of hypoxia by reoxygenation resulted in a return of the acetylcholine sensitivity to normal within the next minute or two.

The cardiac slowing and asystole sometimes seen in acute hypoxia may be related to a similar potentiation of acetylcholine action. It is possible that the apparent increase of cardiac sensitivity to acetylcholine is in reality the result of continued presence during hypoxia of endogenous acetylcholine in increasing amounts summing with that injected.

## 57 THE EFFECT OF CERTAIN SYMPATHOLYTIC AGENTS ON THE CORONARY BLOOD FLOW OF THE DOG

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HAROLD W CHRISTY, M D (BY INVITATION), CHICAGO, ILL

The effect of tetraethylammonium bromide and Dibenamine on the coronary blood flow of dogs was investigated using a modified Morawitz cannula technique for measuring the coronary sinus outflow. The conditions of administration approximated those used in ordinary clinical practice. With tetraethylammonium bromide there was no influence on the coronary sinus outflow while the drug was being injected. After the intravenous infusion was completed, a small increase of the flow was usually observed. The drug had no effect on the characteristic coronary vasodilator action of adrenalin. Intramuscular injection of tetraethylammonium bromide had no detectable influence on the coronary blood flow over a period of several hours. When Dibenamine was given by venoclysis, no change was seen in the coronary flow during, or for several hours after, administration. With the doses used the peripheral vasoconstrictor action of adrenalin was either reversed or nullified. The typical coronary vasodilator action of adrenalin was not affected by Dibenamine. In dogs that had received Dibenamine, stimulation of the peripheral ends of the cut vagi resulted in a modified response. It is apparent that the action of these sympatholytic agents on the coronary blood flow is distinctly different from their action on the blood flow in the extremities. Since these animals were all normotensive it was not surprising to find that there was only a moderate decrease in systolic blood pressure during the administration of tetraethylammonium bromide and Dibenamine. The fact that these sympatholytic agents which are capable of markedly increasing the blood flow of the limbs did not alter the coronary blood flow is of clinical interest.

## 58 DIBENAMINE

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A study was made of the effect of Dibenamine (dibenzyl beta chloroethyl amine) on blood pressure and the peripheral arterial system. Toxic effects were noted. The drug is a tertiary amine and is sympatholytic and adrenergic in action.

Dibenamine was given intravenously to six patients. Five of the patients had essential hypertension and one had normal blood pressure. Skin temperatures were determined on four of the patients after the administration of Dibenamine. The first patient studied received a dose of 2.5 mg per kilogram and the other five received a dosage of 4.0 mg per kilogram.

Dibenamine caused a marked orthostatic hypotension lasting from five to twenty-four hours in all six patients. It also caused a rise in the skin temperature of the lower extremities. The maximum rise in temperature occurred within three to five hours after giving the drug and lasted for from five to forty-nine hours. The vasodilator effect upon the skin lasted longer than the effect upon the blood pressure. Toxic reactions consisted of nausea and vomiting in one case and thrombophlebitis in another. Marked vertigo and faintness occurred in all patients on assuming the standing position. These symptoms disappeared upon reclining. Contraction of the pupils occurred in all. Nasal congestion occurred in five, and two patients complained of dryness of the mouth.

## 59 THE EFFECTS OF DIHYDROERGOCORININE ON THE PERIPHERAL CIRCULATION IN MAN

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 BYARD T. HORTON, MD, AND GUSTAVS A. PETERS, MD (BY INVITATION)  
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The effects of the intravenous administration of dihydroergocorinine (DHE 180), an ergotonic alkaloid on skin temperatures, blood pressure, heart rate and peripheral blood flow were studied in twenty human volunteers. The action of the drug is chiefly sympatholytic and therefore the drug is considered to be a vasodilator. It was administered to six patients by intravenous infusion in a solution containing 0.5 mg of dihydroergocorinine per 100 cc of physiologic saline solution and to fourteen patients by a single intravenous injection. The total dosage varied from 0.25 to 0.4 mg. Control values for skin temperatures, blood pressure, heart rate, and blood flow were determined before the drug was given, and the observations were again recorded at regular intervals for a period averaging sixty-five minutes after administration of the drug. The blood flow was determined by means of a venous occlusion plethysmograph with a compensating spirometer recorder. Skin temperatures were recorded by means of thermocouples applied to the skin over the forehead, over the right and left deltoid muscles, and over the right and left quadriceps femoris muscles.

Dihydroergocorinine produced an overall average increase in peripheral blood flow of 95 per cent in the upper extremities and 68 per cent in the lower extremities in nineteen of twenty cases. In spite of the increase in blood flow in the extremities, the skin temperatures were slightly decreased even during the maximal increase in blood flow.

The blood pressure fell in the two hypertensive patients after administration of dihydroergocorinine. The decrease of systolic pressure was 58 mm Hg in one case and 30 mm Hg in the other, while the diastolic pressure fell 18 mm Hg in the former and 10 mm Hg in the latter. In normotensive subjects there was no significant change in blood pressure.

The heart rate decreased in every case with an average reduction of 13 beats per minute.

Side reactions were more frequent than had been reported by other investigators even with lower dosage. Nasal congestion, nausea, headache, flushing, an urgency for urination and vomiting were the side reactions observed.

## 60 RELATION OF TOBACCO SMOKING TO DISEASES OF THE RESPIRATORY AND CIRCULATORY SYSTEMS

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In order to have a satisfactory picture of *normal* smoking habits, we made a house to house survey in every census tract of the city of Columbus, Ohio, with a verbal questionnaire on individual smoking habits of persons over 20 years of age. About 1,000 persons of each sex and color were interviewed and the results summarized by age groups and city districts (suburban, intermediate and slum). The details of this survey will serve as a useful basis of normal smoking habits in future studies when they are published in detail.

The smoking habits of persons dying of buccal and respiratory tract cancer in Cincinnati and Detroit were compared with those of the Columbus controls and the following differences noted: (1) The incidence of cigar and pipe smok-

ing was very significantly higher among both the buccal and the respiratory tract male cancer victims than among men of corresponding ages in Columbus, while the incidence of nonsmokers was significantly lower than among the proper Columbus controls. Cigarette smoking incidence did not differ significantly from the Columbus standard for either these male or female cancer victims.

With individuals suffering from pulmonary tuberculosis in Dunham Hospital (Cincinnati), the pipe and cigar smoking habits of men were found to be similar to those of the proper Columbus controls, but the incidence of cigarette smoking was significantly higher than normal among both male and female patients. Studies on smoking habits of pneumonia victims are not yet far enough along to report.

Although tobacco smoking has been strongly indicted in various diseases of the circulatory system, adequate detailed and convincing proof has been lacking up to now. To supply proof which could be quantitatively essayed on a mathematical basis, we mailed questionnaires on tobacco smoking habits to the next of kin or informant named on death certificates of all white persons dying in Cincinnati from coronary heart disease during the two years 1946 and 1947. From the returned questionnaires the following findings were obtained: (1) all white victims under 40 years of age were cigarette smokers, (2) the heavy preponderance of cigarette smokers gradually decreased to near the expected normal by the age of 70 years, but by then pipe and cigar smoking was significantly above normal, (3) at all ages the percentage of nonsmokers was very significantly below that found among appropriate Columbus controls.

This survey is being extended to include peptic ulcer and certain other states in which nicotine effects on the autonomic ganglion cells might play a role.

It does seem clear that cigar and pipe smoking is significantly related to cancer of the air passages and lungs, while cigarette smoking is significantly related to infectious diseases of the respiratory tract. Cigarette smoking below 65 years of age and pipe or cigar smoking above that age are significantly related to fatal attacks of coronary heart disease.

## 61 FACTORS INFLUENCING THE T WAVE OF THE ELECTROCARDIOGRAM, AN EXPERIMENTAL STUDY EMPLOYING INTRACAVITARY AND EPICARDIAL LEADS

### I EFFECTS OF HEATING AND COOLING OF SUBEPICARDIAL AND SUBENDOCARDIAL LAMINA

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(INTRODUCED BY HAROLD FEIL, M.D.)

Only recently has the question been raised as to the contribution of depolarization and repolarization of the subendocardial lamina to the genesis of the electrocardiogram. The employment of intracardiac catheter electrodes has facilitated the study of the electrical influence of the myocardium. In the present study this technique was employed to investigate the genesis of electrical events established by changes in the rate and direction of repolarization of subepicardial and subendocardial lamina. These changes were produced by heating and cooling the epicardium and the endocardium. Intra- and extracardiac (epicardial) exploring unipolar electrodes were used. The method of changing the temperature of the subendocardial surface relative to that of the epicardium consisted of introducing physiologic saline at various temperatures through in-dwelling intracavitary catheter electrodes. Epicardial thermal changes were

produced by local application of cold and hot saline soaked pads and ice cubes of various sizes. The effects were immediate with rapid recovery occurring in ten seconds to two minutes.

Negative T waves occurred where the exploring electrode immediately subtended areas which had relatively or absolutely retarded repolarization. Positive T waves occurred where the exploring electrode faced areas which had relatively or absolutely accelerated repolarization.

It was found that negative T waves occurred

A In the cavity (1) when the endocardium was cooled (2) when the endocardium was not altered, but the epicardium was heated

B On the epicardium (1) when the epicardium immediately under the electrode was cooled, (2) when the endocardium subjacent to the epicardial exploring electrode was heated (3) when the endocardium of the opposite wall was cooled

Positive T waves occurred

A In the cavity (1) when the endocardium was heated, (2) when the epicardium was cooled

B On the epicardium (1) when the subjacent epicardium was heated, (2) when the subjacent endocardium was cooled

Direct evidence has been obtained that changes in the electrical state of the subendocardial muscular lamina modify the form of the electrocardiogram. Changes in the T wave can be produced by changing the rate and order of endocardial epicardial lamina repolarization.

The relation of our results to the modern concept of altered repolarization in an extensive conducting medium is discussed. The spatial relation of the exploring electrode to a theoretical surface at the junction of the normal and altered areas is used to account adequately for the findings.

The practical value of these observations is discussed.

The present results confirm and extend previous observations on the contribution of the subendocardial myocardium to the electrocardiogram.

## 62 A DIRECT WRITING OSSILOGRAPH FOR RECORDING DATA OBTAINED BY THE METHOD OF CARDIAC CATHETERIZATION

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With the cooperation of the Brush Development Company of Cleveland we have adapted their direct writing six channel Ossilograph for recording simultaneously such data as the electrocardiogram, femoral arterial pressure, intracardiac or pulmonary arterial pressure, the respiratory cycle time in seconds, and the Ballistocardiogram. By using strain amplifiers, pressure transmitters, and strain gauges we have found that this system records accurately variations in blood pressure as checked with the Hamilton manometer. Furthermore the system has certain advantages such as linearity of range scale, mobility, and direct visualization of phenomena being recorded and it is simple to calibrate. Data as recorded by the Ossilograph and derived from observations on patients with heart disease are presented in a series of lantern slides.

### 63 A COMPARATIVE EVALUATION OF EXTREMITY AND PRECORDIAL LEADS IN THE DIAGNOSIS OF ACUTE MYOCARDIAL INFARCTION

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(INTRODUCED BY EDMUND F. FOLLY, M.D.)

There has been a great deal of controversy concerning the relative merits of standard leads I, II, and III versus extremity potentials  $aV_R$ ,  $aV_L$ , and  $aV_F$ . In addition the decision whether to use a precordial position with CF, CR, or V as the indifferent electrode has not been decided. Our purpose was to evaluate and compare these leads in recent acute myocardial infarction.

Fifty patients were studied, of whom twenty-five were shown by serial electrocardiographic, clinical, and autopsy data to have had an acute myocardial infarction. Of these twenty-five patients, seven showed equal diagnostic findings in standard leads, extremity potential leads, and precordial leads. Six had anterior involvement and one posterior. There were five in whom the precordial leads showed the most significant findings. The extremity leads I, II, and III and  $aV_R$ ,  $aV_L$ , and  $aV_F$  were not normal but showed no acute involvement as manifested by S-T segment elevation or T-wave inversion. In five the extremity potentials  $aV_R$ ,  $aV_L$ , and  $aV_F$  were more helpful in making a diagnosis than were the standard leads. In these the precordial leads were very useful also. Four patients with posterior infarctions showed the best changes in the extremity leads, precordial leads not being of much aid. Four others with posterior involvement were definitely aided by precordial leads.

The second study concerning the use of the left foot, the right arm, or the central terminal as the indifferent electrode position did not reveal significant differences. The voltages were not the same, but for clinical purposes they so closely resembled one another that we felt one indifferent electrode position to be as useful as another. When an S-T segment was elevated or a T wave inverted or a deep Q wave present in  $CF_4$ , for example, it was similarly altered in  $CR_4$  and  $V_4$ .

In conclusion the extremity potentials  $aV_R$ ,  $aV_L$ , and  $aV_F$  show diagnostic changes more frequently than the standard leads I, II, and III. No significant clinical differences were seen between CF, CR, or V precordial leads in the diagnosis of acute myocardial infarction.

### 64 THE ELECTROCARDIOGRAPHIC RESPONSE TO THE ANOXEMIA TEST IN PATIENTS WITH HYPERTHYROIDISM

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Observations of an anoxemia test that had been performed on a patient taking thyroid extract revealed a positive test. The test became normal on discontinuing thyroid. This led to the study of nine patients with clinical hyperthyroidism. All nine patients were selected carefully so that the symptoms were of recent duration, the basal metabolic rates on repeated tests were over 30 per cent, the age was under 40 years, and repeated examination revealed no evidence of cardiac disease.

It was then presumed that we were dealing with individuals whose hearts were normal except for any influence the hyperthyroidism might have had.

In all nine patients positive tests were observed. The S T segments became depressed below 1 mm and T wave inversion especially in Leads I II, CF<sub>1</sub> and V were seen. As the hyperthyroidism was controlled by medical management propylthiouracil or surgery, the response to the anoxemia test was not as dramatic. Further studies are in progress at this time.

This test with the production of anoxemia has shown objectively the damaging effect of the hyperthyroidism. By implication one might anticipate that patients with normal thyroid glands taking thyroid extract for purposes of reduction would show similar changes.

## 65 ELECTROCARDIOGRAPHIC CHANGES FOLLOWING MEALS IN PATIENTS WITH ANGINA PECTORIS

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(INTRODUCED BY JOHNSON McGUIRE, M.D.)

Standard mixed meals, consisting of 90 grams carbohydrate, 40 grams protein and 40 grams fat were given to thirty-two patients with angina pectoris. Electrocardiograms were taken on all patients before meals and thirty minutes following the meal. In some cases electrocardiograms were also taken sixty minutes and ninety minutes following meals. Warm food and warm liquids were used in each meal. In approximately 25 per cent of the patients there occurred a  $\frac{1}{2}$  to  $\frac{3}{4}$  mm depression of the S T segment and development of or increase in existing concavity of the S T segment. In patients showing this S T change following meals, repeat electrocardiograms showed these findings to be consistent. Fourteen normal controls in the same age group exhibited no similar change.

The effect of meals on the electrocardiogram in these normal controls was similar to that reported by Simonson, Alexander, Henschel and Keys in normal individuals. These changes consisted of the following: a significant increase in heart rate, decrease in Q T interval, decrease in amplitude of the T wave, increase in QRS amplitude and shift of the electrical axis toward the left. The patients with angina pectoris exhibited these changes also.

The electrocardiogram changes induced by the ingestion of food in the anginal patient suggests the possibility of employing this or similar methods as an additional aid in detecting coronary insufficiency.

## 66 CIRCULATION TIMES FOR ANGIOCARDIOGRAPHY

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(INTRODUCED BY DON C. SUTTON, M.D.)

The diagnostic justification for the performance of angiocardiology frequently depends upon obtaining a satisfactory levoangiocardigram. This has been difficult to do when using the method of Robb and Steinberg because of difficulty in determining in advance when the contrast media will fill the left heart and aorta. Previous methods of determining circulation times prior to angiocardiology have not been totally satisfactory. Experiments in circulation time conducted by our group confirmed the work of others who had noted that increasing the volume injected decreased the circulation time. In a

group of twenty adults, none of whom had valvular cardiac lesions, increasing the volume of saline diluent from 5 to 50 ml decreased the ether aim to pulmonary capillary circulation time by a mean of 1.3 seconds. A similar increase in volume decreased the sodium cyanide aim to carotid sinus circulation time by a mean of 6.3 seconds. The increased volume of material rapidly injected into the aim vein accelerated the circulation time to the right heart and *through* the lungs and left heart as well. Angiocardiograms timed on the basis of circulation times determined by the use of quantities of material equal to the contrast media used (50 ml in this series), and injected in a manner identical to the media, produced satisfactory levoangiocardigrams.

## 67 A COMPARATIVE STUDY OF PERIPHERAL AND DIRECT INTRACARDIAC ANGIOCARDIOGRAPHY

GEORGE C. SUTTON, M.D., GEORGE E. WENDEL, M.D., AND  
HARRY E. GRANT, M.D., CHICAGO, ILL.

(INTRODUCED BY DON C. SUTTON, M.D.)

Two methods are available for the performance of angiocardiography. The first method, utilizing the injection of the contrast media into a peripheral aim vein, fails to provide satisfactory visualization in a number of patients. This occurs most frequently in adults who possess thick, muscular thoracic walls or dense mediastinal or lung lesions which lessen the contrast of the dye filled structures. Direct intracardiac injection of the contrast media by means of a catheter introduced into the jugular vein in the neck and advanced to the right auricle is the second method. A comparative study of these two procedures has been made by the group working in Pieble Laboratory.

## 68 THE APPEARANCE OF NORMOBLASTS IN THE PERIPHERAL BLOOD IN PATIENTS WITH PULMONARY EMBOLISM

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(INTRODUCED BY MAURICE HARDGROVE, M.D.)

Greater attention is being directed toward the study of the peripheral blood in cardiac failure and associated conditions. The quantitative changes, including the cellular elements, have been observed and reported. References to the qualitative changes of the cellular elements are extremely infrequent. During the past two years we have carefully studied the peripheral blood of all cases of pulmonary embolism that have come to our attention. We are reporting six cases of pulmonary embolism or thrombosis, with resultant right heart failure and the appearance of normoblasts in the peripheral blood stream. Other less noteworthy qualitative changes were observed, such as the findings of reticulocytes and polychromasia. All cases were autopsied, three of which showed extramedullary hematopoiesis. The explanation for the outpouring of normoblasts is not known. Acute severe anoxia would seem to be the most plausible etiologic factor. However, the anoxia produced by sudden altitude changes has not been reported to release normoblasts to the peripheral blood. In view of this, it is felt that other factors must play a role. Some of the additional factors postulated include tissue destruction and hemolysis.

(To be concluded in the December issue)



## PARENTERAL NUTRITION

### VII METABOLIC STUDIES ON PUPPIES INFUSED WITH FAT EMULSIONS

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THE preparation of fat emulsions with high caloric concentrations for parenteral supplementation or replacement of oral feeding has been studied intermittently by various workers for over fifty years.<sup>1-4</sup> Reports have indicated varying conclusions as to the practicability of preparing an emulsion of fat that can be given intravenously for nutritional purposes. Work done in this laboratory over the past six years has shown that fat emulsions suitable for intravenous administration can be prepared and that the calories in such preparations are available for energy requirements when the emulsions are given intravenously.<sup>20</sup>

The experiments described in this paper were undertaken in an effort to study the effect of intravenous fat emulsions upon growth, nitrogen balance, and selected biochemical and hematologic functions. Two litters of puppies were used to determine (1) whether young and rapidly growing animals, with a consequently high caloric requirement, can utilize an emulsion of fat given intravenously (2) whether daily infusion in large amounts (15 to 100 Gm. of fat per kilogram of body weight) of the emulsion now used in this laboratory<sup>20</sup> will lead to either histologic, hematologic, or metabolic injury.

### EXPERIMENTAL

The puppies were housed in an air conditioned room in individual metabolism cages permitting accurate collection of urine and feces. They were weighed daily. Nitrogen balance studies were conducted on a three-day interval scheme. The animals were maintained three days on a new regime and the nitrogen intake and output were then measured for each of three consecutive days and the balances were computed for each dog. Nitrogen determinations on diet and excreta were done by the macro Kjeldahl method.

Hematologic studies were done on blood collected in a dry syringe from a foreleg vein and transferred to a tube containing balanced oxalate. Hemoglobin determinations were done by a direct photometric method.<sup>6</sup> During infusion periods blood was drawn for hematologic and chemical studies just before the start of an infusion; this was usually twenty-four hours after the last infusion.

From the Department of Nutrition, Harvard School of Public Health and the Departments of Biological Chemistry and Legal Medicine, Harvard Medical School.  
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Emulsion No. 35 composition in grams: coconut oil 300, phosphatide fraction BF 30, dextrose 35, water 634. For details of preparation see reference 5.

Total serum protein and serum albumin were determined by the method of Howe, serum nonprotein nitrogen by the nesslerization procedure of Koch and McMeekin,<sup>8</sup> and serum bilirubin by the diazotization procedure of Malloy and Evelyn.<sup>9</sup> Serum total and free cholesterol determinations were done by the method of Schoenheimer and Sperry.<sup>10</sup>

Bromsulfalein excretion was determined by the method previously described by McKibbin<sup>11</sup> and plasma prothrombin by the method of Link.<sup>12</sup> Plasma volumes were determined by the method of Hopper and co workers<sup>13</sup> and red blood cell fragility by the method of Wintrobe<sup>14</sup> or Parpart.<sup>15</sup> Fecal lipids were determined as follows. Collections of the twenty four hour feces for each dog were mixed in a Waring blender with the additions of 95 per cent ethyl alcohol and concentrated hydrochloric acid until acid to Congo red. An aliquot of the mixture was dried in vacuo and extracted with dry chloroform for twenty four hours in a Soxhlet extractor. The chloroform was then removed in vacuo, the residual fat taken up in low boiling (40 to 60° C) petroleum ether and filtered into tared beakers. The ether was removed and the chloroform extractable, petroleum ether soluble lipid determined by weighing

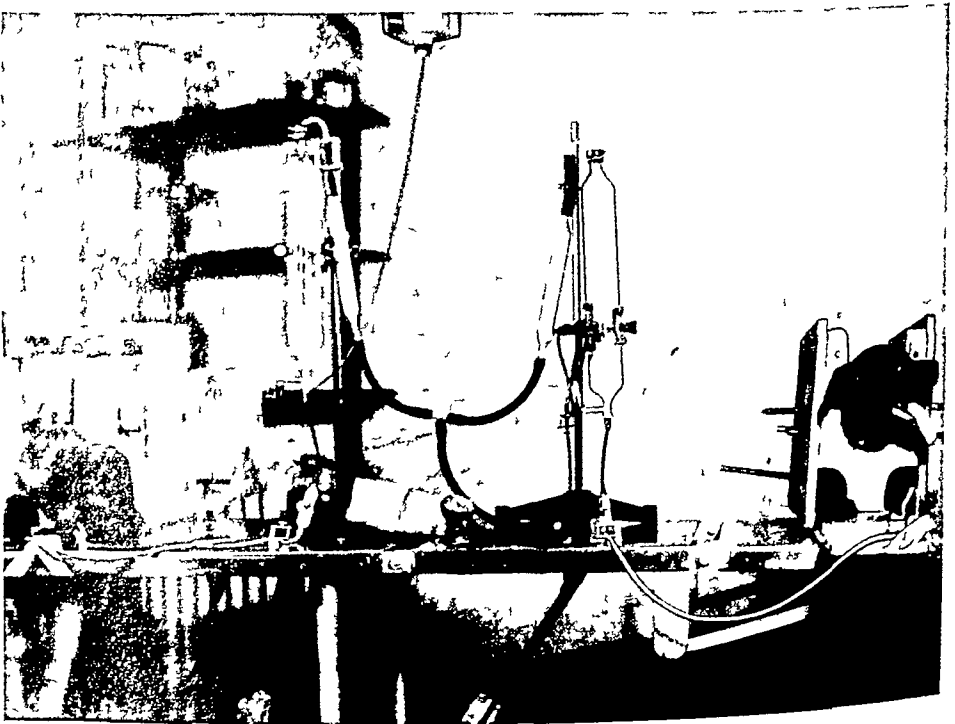


Fig 1—A photograph of the apparatus used for infusion of animals

Infusions of fat emulsion were carried out by placing the animal in an adjustable animal box in an upright position. A foreleg was secured with adhesive tape and after preparing the area for injection, the needle (No 20 or No 22) attached to the infusion set was inserted into a vein on the dorsum of the leg. When the needle had been taped securely in place the infusion could run for several hours without discomfort to the animal. For accurate measurement of infusion rates we found it advantageous to use an infusion set consisting of a graduated cylinder as a reservoir, a Murphy drip bulb, and a closed system with positive air pressure maintained by an attached blood pressure cuff and aneroid manometer (Fig 1).

The composition of the various diets fed the puppies in these studies is shown in Table I

TABLE I COMPOSITION OF DIETS USED FOR EXPERIMENTS I AND II

DIET	D1 (GM %)	D2 (GM %)	GAMES DOG MEAL† (GM %)	
Lactin (crude)	20	10	Protein	25.1
Liver extract Wilson's 1:20	2	2	Ash	8.0
Yeast, Anheuser-Busch strain K	10	10	Fat	6.0
Games Dog Meal	0	78	Fiber	2.9
Dextrose	65	0	Nitrogen	19.6
Salt mixture IV	3	0	Moisture	7.2

J Biol Chem 158:4-9, 1941

†Manufacturer's analysis

*Experiment 1*—A litter of eight Labrador retriever puppies reared in this laboratory was weaned at 6 weeks of age and during two weeks were gradually transferred to diet D1. The animals were fed *ad libitum* the daily food intake was measured, and nitrogen balances were determined at intervals.

After an initial control period four puppies (Nos. 90, 906, 907 and 909) were started on daily infusions of a 30 per cent coconut oil emulsion stabilized with a 1 per cent phosphatide preparation—Emulsion No. 35.<sup>5</sup> Two pups, Nos. 904 and 908, were kept as control animals and fed ration D1 *ad libitum*.

The initial infusions were given rapidly at a rate of 2 to 4 ml per minute and led to two immediate toxic reactions. On the first day of infusions the animals showed evidence of vasomotor collapse with pale mucous membranes and sometimes with drowsiness and defecation. This reaction occurred after no more than 25 ml of the emulsion had been given. After five to ten minutes with the infusions continuing at the initial rate the animals recovered. The reaction did not reoccur on successive days. The second complication of immediate importance was the occurrence of vomiting and anorexia. Vomiting appeared either as a result of rapid infusion or after long continued infusion at a slow rate. Since appetite is determined in part by caloric requirement the infusion of such a large amount of fat (10 Gm of fat per kilogram of body weight) to supply calories would be expected to decrease the oral intake of food to the point of serious limitation of protein, vitamins and minerals. These limitations would in turn aggravate the anorexia and if continued for any time would give rise to serious deficiency complications. When the limitation of oral intake became apparent (after five to seven days) the infusions were stopped for five days and the animals, including the two control pups, were transferred to a diet of Games Dog Meal to allow them to regain nutritional equilibrium. Infusions in the experimental animals were commenced again at a rate of 3 to 5 ml per minute but in a total quantity to supply only 15 Gm of fat per kilogram of body weight per day. This was well tolerated. After six days the quantity of emulsion infused was increased to 3 Gm of fat per kilogram. At this level of infusion growth was good and the animals were in positive nitrogen balance while receiving approximately 20 per cent of their caloric requirement as intravenous fat. Figs. 2, 3, 4, and 5 show the data on oral food consumption, infusion of fat, growth, hematologic data, and nitrogen balance obtained on these four puppies. The hematologic data reveal a moderate but significant fall in hemoglobin, red blood cell count

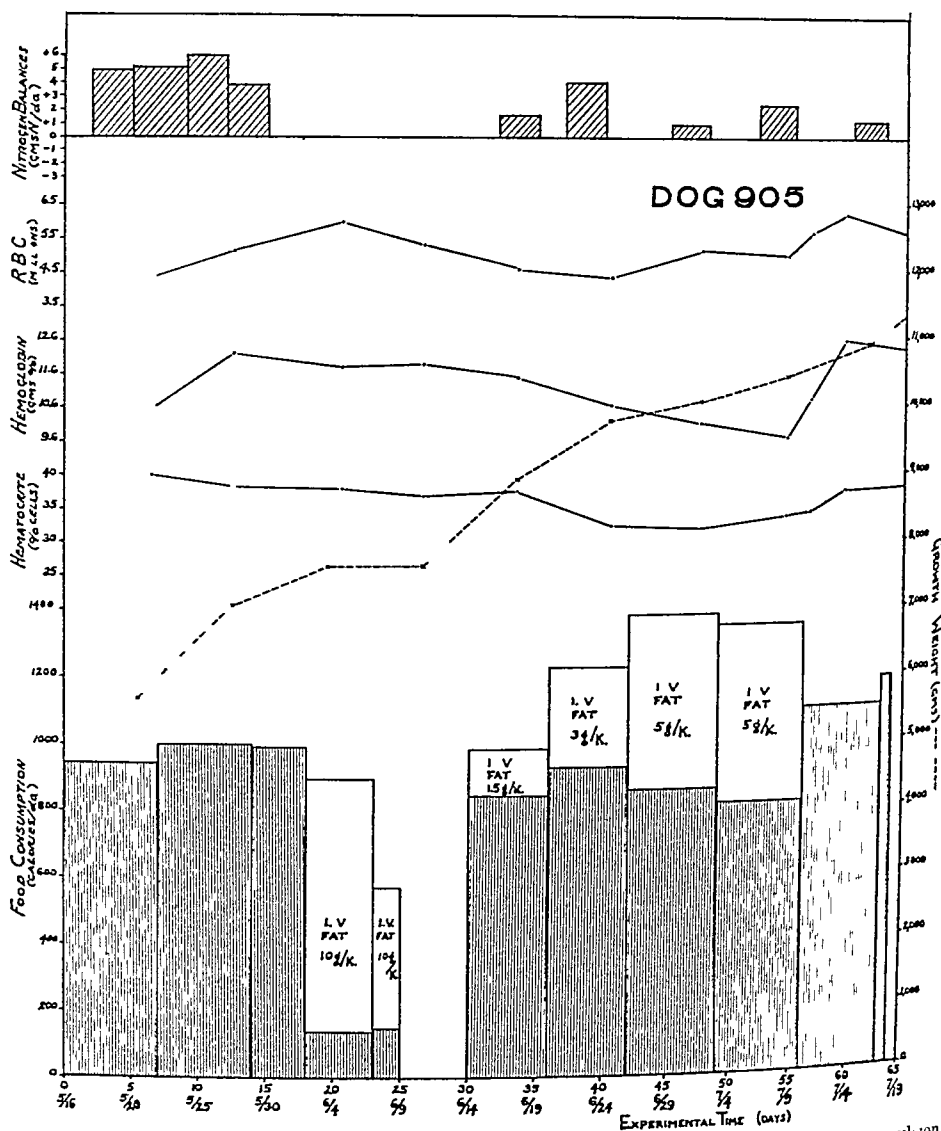


Fig 2—Data obtained from Dog No 905 Experiment 1—infusion of 30 per cent fat emulsion.

and hematocrit. Similar blood changes have been reported from this laboratory by Collins and co-workers.<sup>10</sup> It is of interest to note that the anemia produced is self-limited even though infusions continue. The data presented on Pup No 909 in Fig 5 particularly emphasizes this point.

The puppies received 3 Gm of fat per kilogram of body weight per day for six days and then the amount of infused fat was increased to 5 Gm of fat per kilogram. At this higher level the results obtained were not so uniform. Puppies 905 (Fig 2), 906 (Fig 3), and 907 (Fig 4) grew well and remained in positive nitrogen balance, but Puppy 909 (Fig 5) grew poorly and was for a time in negative nitrogen balance. After twenty-one days on this infusion level of 5 Gm of fat per kilogram of body weight, Pup 909 was

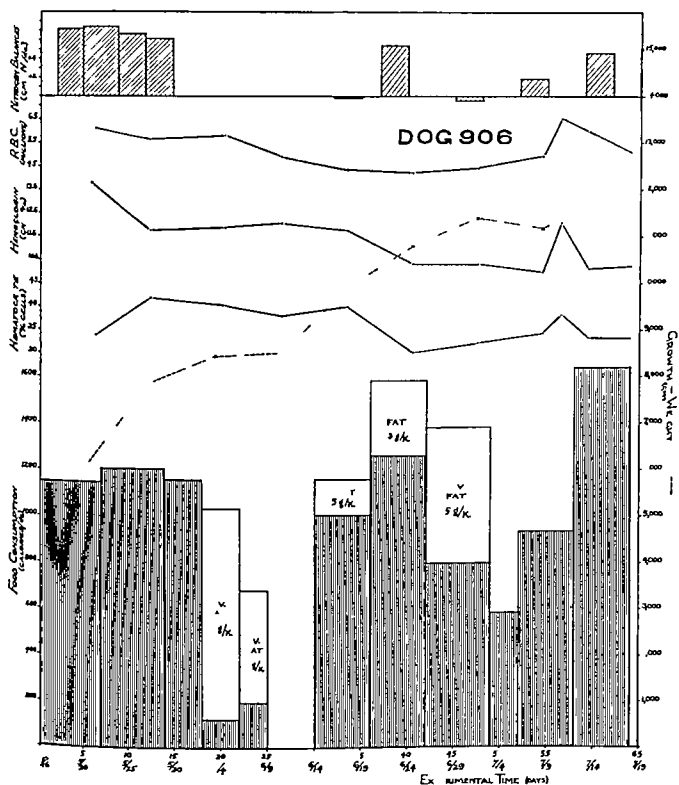


Fig 3—Data obtained from Dog No 906 Experiment 1—infusion of 30 per cent fat emulsion

again given 10 Gm of fat per kilogram per day and continued on this regime for an additional twenty four days with the object of determining the effect of continued large infusions of fat. At this extremely high level of fat infusion the blood lipid level remained high even twenty four hours after the last infusion indicating that the infusions were surpassing the utilization and storage rates. There was occasional vomiting during or immediately after the infusions. Oral intake of food remained essentially constant for the first two weeks following this high level of fat and then progressively diminished. It will be noted that there was a steady gain of weight with an increasingly positive nitrogen balance, until the seventy fifth day of the experiment. After

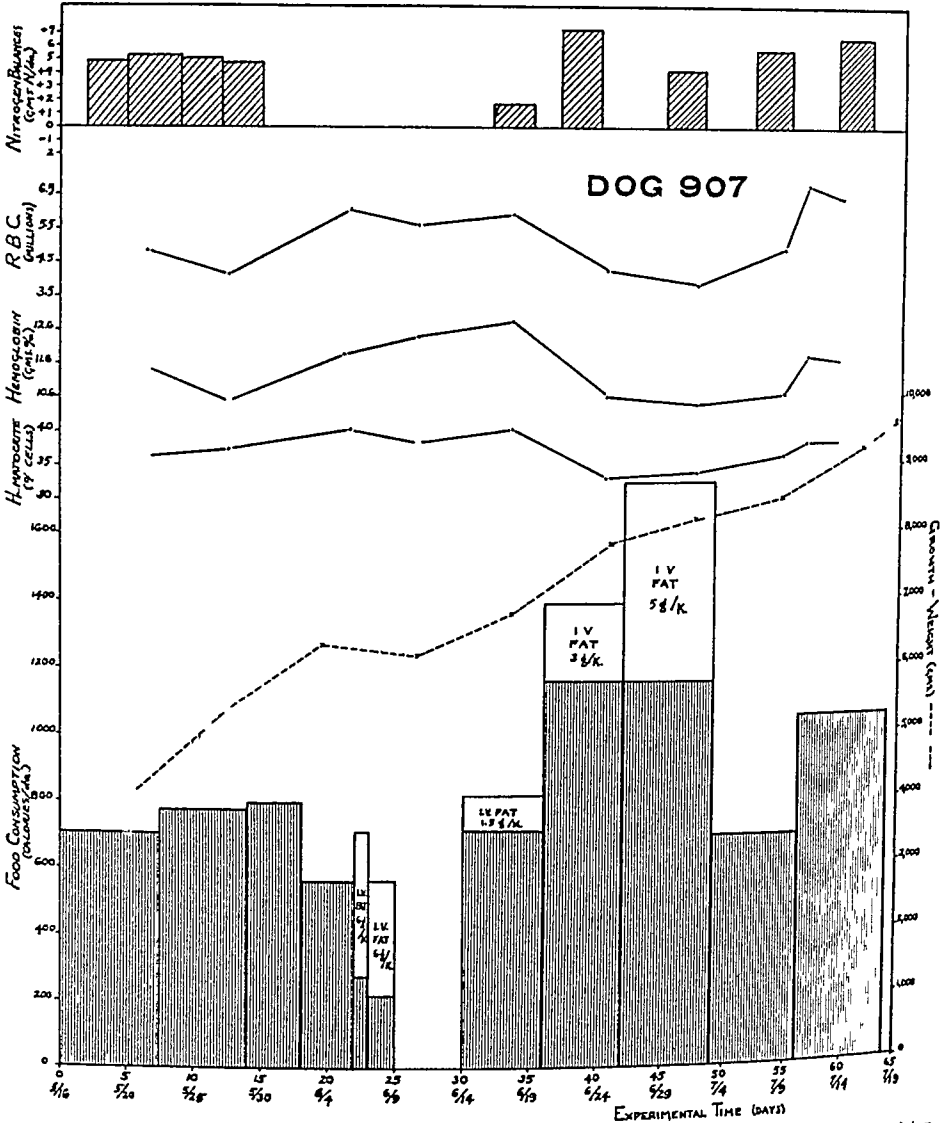


Fig 4—Data obtained from Dog No 907 Experiment 1—infusion of 30 per cent fat emulsion

this time, the oral intake fell to inadequate levels, the animal became thin and gaunt and died on the ninety-first experimental day. Over the course of the experiment, this animal had received 3,030 Gm of fat by vein. The terminal rise in blood values was probably due to dehydration and hemoconcentration. The precise cause of this dog's death is not known, though the anorexia and inadequate intake of essential nutrients were no doubt important factors. The loss of fat tolerance as indicated by progressive abnormality of tolerance curves may be of significance.

The two control pups (Nos 904 and 908) which had been maintained on a diet of Games Dog Meal grew well, showed a normal blood picture, and except for occasional respiratory infections, appeared in good health.

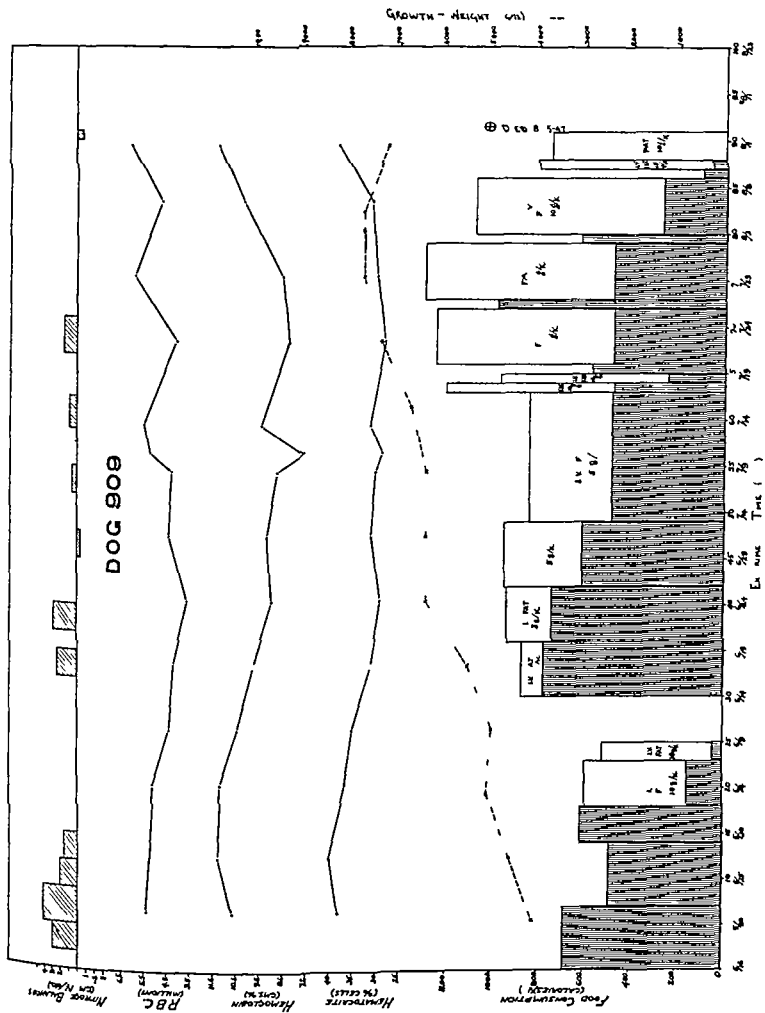


Fig. 3.--Data obtained from Dog No. 909. Experiment 1--Infusion of 30 per cent fat emulsion

*Pathology of Animals in Experiment 1* Table II indicates the relationship of the infusions to the time of autopsy of this group of animals. At post mortem the animals in Experiment 1 showed no gross changes. Microscopic examination of stained sections revealed that the lungs of animals 905 and 907,

TABLE II SUMMARY OF FAT INFUSIONS IN EXPERIMENT 1

DOG	DAYS INFUSED	TOTAL FAT INFUSED (GM.)	DAYS AFTER LAST INFUSION ANIMAL SACRIFICED	COMMENT
904	0	0	---	Control animal
908	0	0	---	Control animal
907	22	647	18	Tolerance good
906	26	1,225	110	Tolerance good
905	33	1,466	20	Tolerance good
909	63	3,030	5 hr	Animal died

infused for thirty-three and twenty-two days respectively, contained occasional accumulations of multinucleated giant cells. These were graded 1+ according to the criteria previously outlined.<sup>5</sup> No granulomatous lesions were found. Fat stains of the liver were not remarkable. The bone marrow showed a moderate degree of hyperplasia. The remaining organs were normal. Animal 909 showed evidence of terminal pneumonia with peribronchial infiltration of inflammatory cells, but neither granulomatous nor giant cell-containing lesions were found. The control animal (No. 904) sacrificed at this time showed similar evidence of pulmonary infection suggesting a viral disease. Infusion animal No. 906 was not sacrificed until one hundred and ten days after the last infusion. The remaining control pup (No. 908) was sacrificed at that time. Neither animal showed any evidence of histologic changes.

*Experiment 2*—With the experience of the preceding study a second experiment was designed to accomplish two purposes: (1) to avoid the immediate toxic reactions and anorexia complicating the first experiment by more judicious adjustment of the rate and amount of infusion and composition of the diet, (2) to study several metabolic functions of the animals in order to determine the effects of both the complete emulsion (Emulsion 35<sup>5</sup>) and the phosphatide emulsifier BF2<sup>5</sup> alone.

A litter of eight mongrel puppies was placed on diet D2 (Table I) at 16 weeks of age. The animal procedures and nitrogen collections were carried out as described for the preceding experiment. In addition to hematologic studies, the following determinations were made at regular intervals: serum total protein and albumin, serum total and free cholesterol, serum nonprotein nitrogen, plasma volume, bromsulfalein clearance, serum bilirubin, and plasma prothrombin concentration.

The eight animals were divided into four groups of two animals each as indicated in Table III. Animals I and II were infused with a 30 per cent coconut oil emulsion stabilized with 3 per cent of a phosphatide fraction (Emulsion 35). Animals III and VI, with an emulsion of 3 per cent phosphatide fraction BF2<sup>5</sup> without added fat, animals IV and V were not infused but were pair-fed with animals I and II respectively. Animals VII and VIII served



as controls, were not infused, and were fed diet D2 ad libitum. The infusions were given at a rate of 3 to 5 ml per minute and animals I and II which received the fat emulsion obtained a total of 2 Gm of fat per kilogram of body weight per day from this source for the first two days, 3 Gm of fat per kilogram for the next seventeen days, and 5 Gm of fat per kilogram for the last twelve days.

TABLE III SUMMARY OF FAT INFUSIONS IN EXPERIMENT 2

DOG	INFUSION	ORAL FOOD	INFUSION TIME (DAYS)	TOTAL COCONUT OIL INFUSED (GM)	TOTAL PHOSPHO- LIPID BF2 (GM)	TIME AFTER LAST INFUSION SACRIFICED (DAYS)
I	30% fat emul sion	Diet D2 ad libitum	31	1.06	1.1	16
II	30% fat emul sion	Diet D2 ad libitum	31	1.486	1.39	16
III	3% BF2	Diet D2 ad libitum	31	0	106	16
VI	3% BF2	Diet D2 ad libitum	31	0	142	16
IV	0	Pair fed with Dog I	4	.292	.29	9
V	0	Pair fed with Dog II	4	.92	.9	5 hr
VII	0	Ad libitum control	0	0	0	0
VIII	0	Ad libitum control	0	0	0	0

The course of this experiment is charted in Figs 6, 7, 8 and 9. Fig 6 presents the data obtained on animals I and II which were infused with the 30 per cent of fat emulsion. The data emphasize again adjustment of appetite and oral food intake to maintain a constant total caloric intake. These two puppies grew well if not better than the ad libitum fed control animals (animals VII and VIII, Fig 9) although they obtained 25 to 30 per cent of their total calories from intravenous fat. Fig 7 shows the data on animals III and VI and it is seen that the phosphatide material BF2 alone had little if any, effect upon appetite or growth.

Nitrogen assimilation was comparable in the infused dogs with that of the control, noninfused animals. In sharp contrast Dogs IV and V, pair fed with Dogs I and II were barely able to maintain their body weight and nitrogen equilibrium (Fig 8). They were, of course, receiving fewer calories to the extent of the calories furnished by the parenteral fat to Puppies I and II. The growth curves of each pair of animals in this experiment have been averaged and are all presented for comparison in Fig 10.

Since Puppies IV and V were behind in growth it was decided to give them large infusions of fat to see whether there would be a prompt gain in weight. Accordingly, on the fifty second day of the experiment a series of four infusions of a 30 per cent fat emulsion amounting to 10 Gm of fat per kilogram of body weight were given. Unfortunately this emulsion was prepared by a different procedure and proved to be unstable in vivo. As Figs 8 and 11 indicate it was immediately toxic. Because of the toxic reactions of this poor batch of emulsion animals IV and V are excluded from the discussion of post mortem changes.

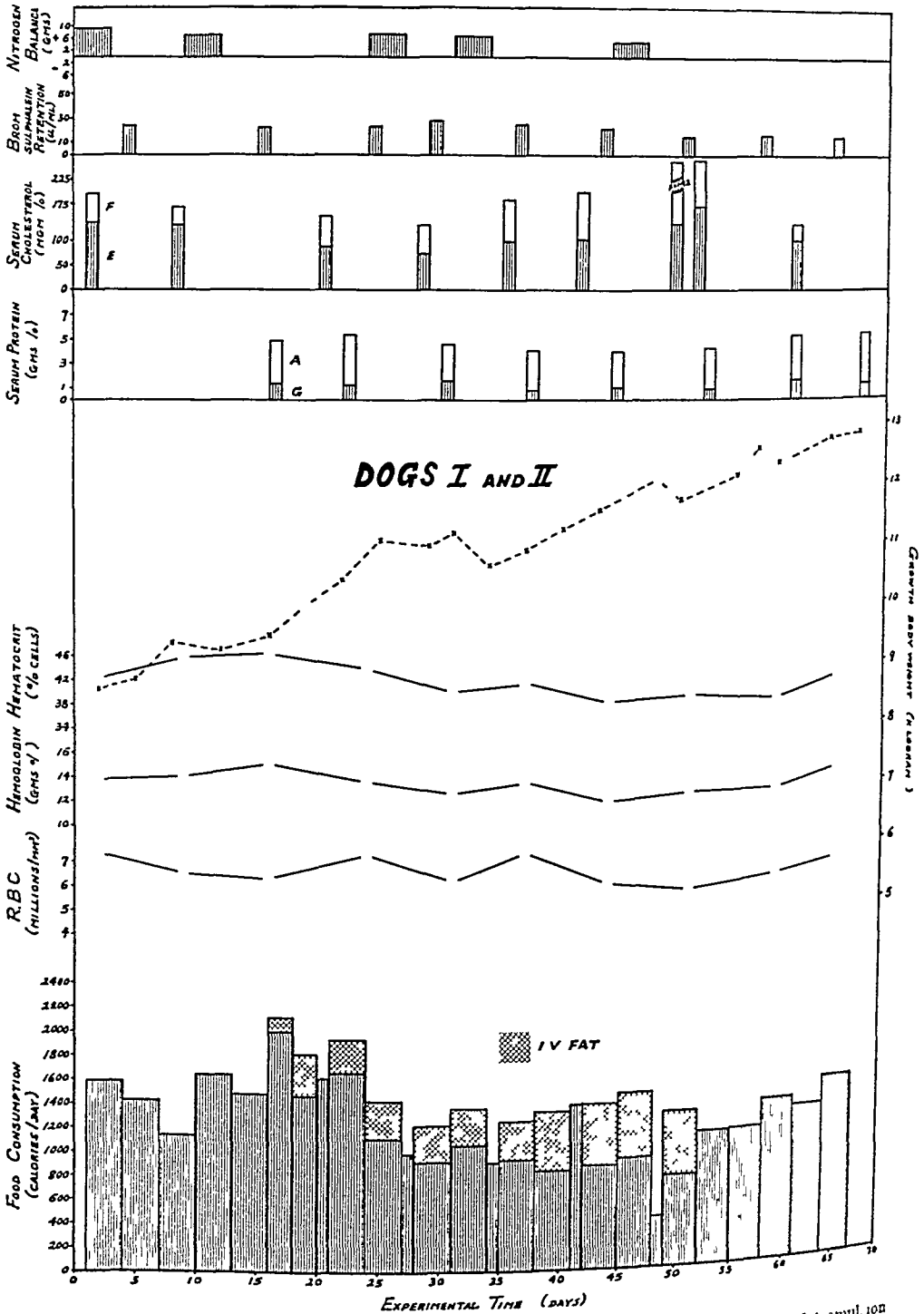


Fig 6 —Data obtained from Dogs I and II Experiment 2—infusion of 30 per cent fat emulsion

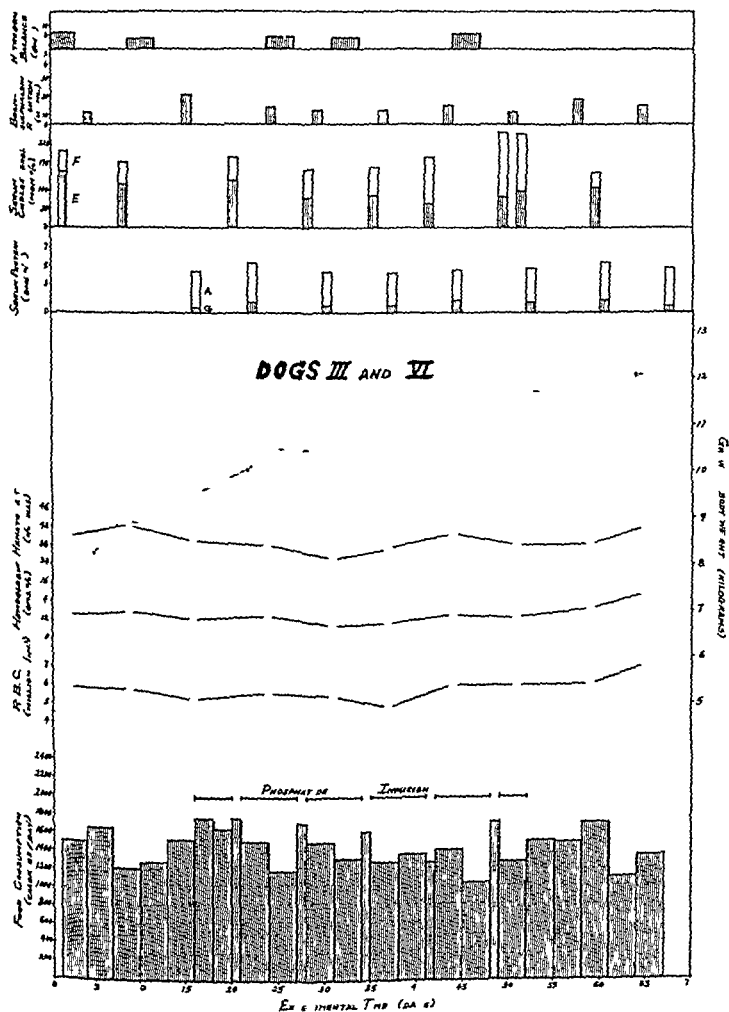


Fig —Data obtained from Dogs III and VI Experiment —infusion of phosphatide stabilizer without added fat

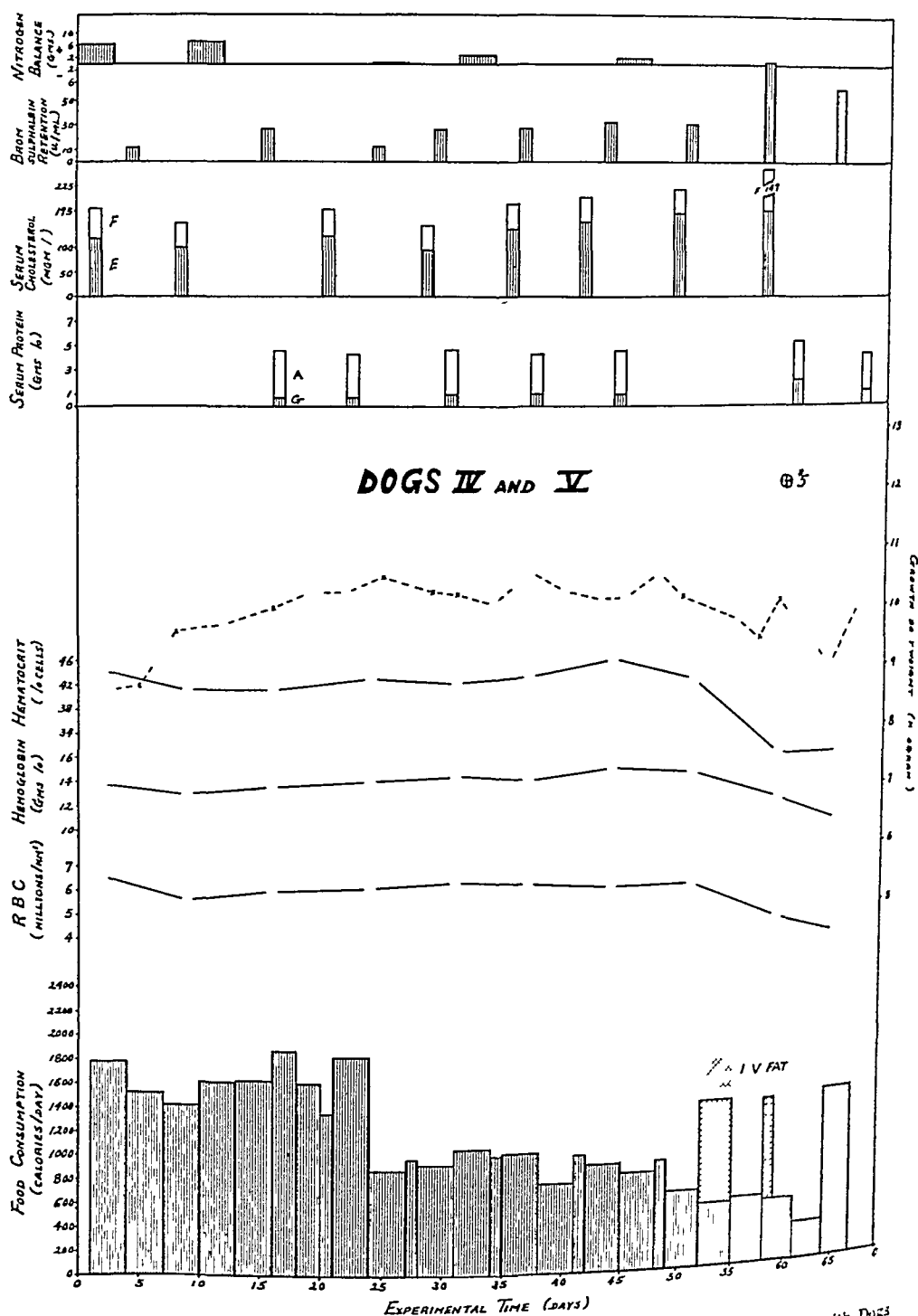


Fig 8—Data obtained from Dogs IV and V Experiment 2—control animals pair fed with Dogs I and II, no infusion

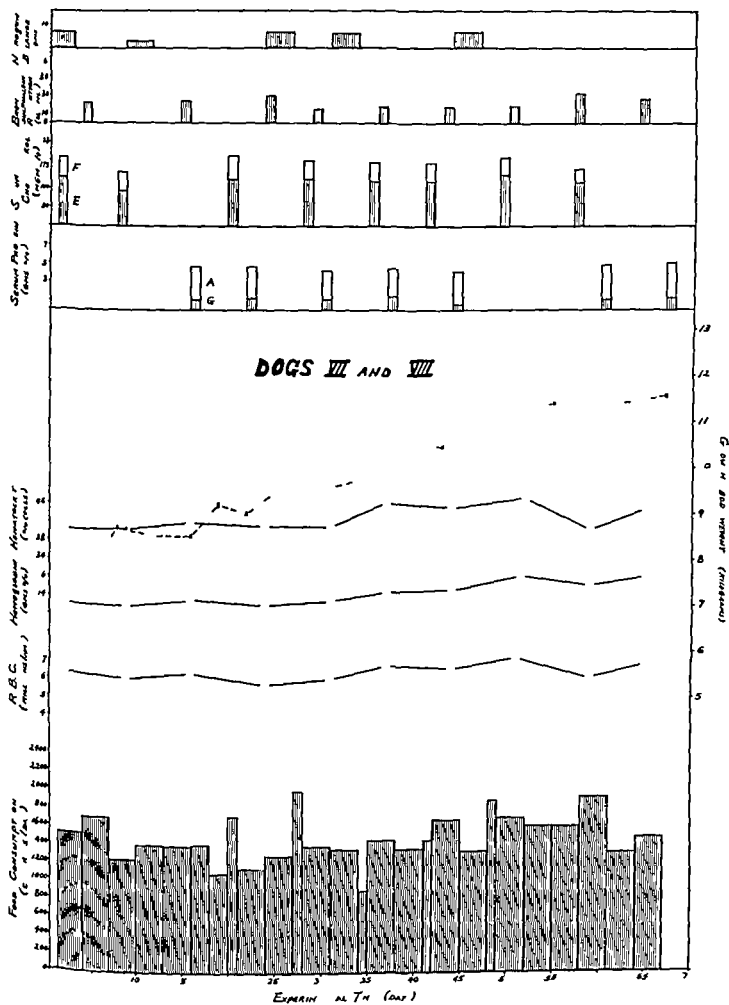


Fig 9—Data obtained from Dogs VII and VIII. Experiment —control animals fed ad libitum no infusion.

## GROWTH CURVES OF PUPPIES

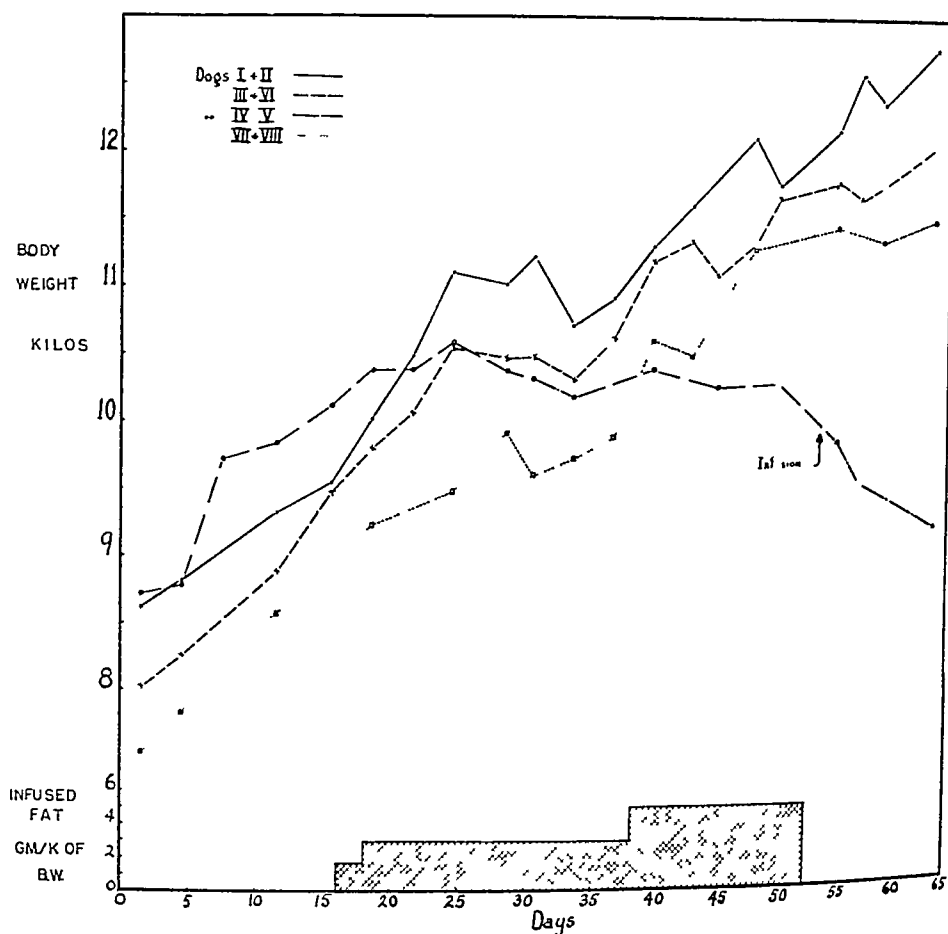


Fig 10—Growth curves of all animals in Experiment 2

Hematologic studies revealed a small but significant and selflimiting fall of hemoglobin, hematocrit, and red blood count during the first two weeks of the infusion period. This change was of the same magnitude in the animals receiving the phosphatide BF2 alone as in those receiving the fat emulsion. Serum bilirubin determinations during this period did not reveal evidences of increased blood destruction.

Determinations of red blood cell fragility revealed consistently decreased resistance of the infused animals' blood cells to hypotonic saline, this seemed to be caused by the phosphatide emulsifier rather than by the fat itself since the effect was no greater in the dogs receiving the coconut oil emulsion than in those receiving only the phosphatide. The mechanism of this effect has not been established.

Neither the serum protein concentration nor the total circulating serum protein calculated from plasma volumes was significantly altered. Serum albumin concentration and total circulating serum albumin were not significantly changed. The serum nonprotein nitrogen remained within normal limits.

An interesting and perhaps significant finding detected in the infused animals was a striking increase in the proportion of free serum cholesterol. This increase became significant two weeks after the start of the infusions and it was more marked in the animals receiving only the phosphatide stabilizer. One week after cessation of infusion the ratio of free to total cholesterol had returned to normal. The serum cholesterol changes in Puppies

### CHOLESTEROL VARIATIONS IN DOGS AFTER INFUSION OF LIPIDS

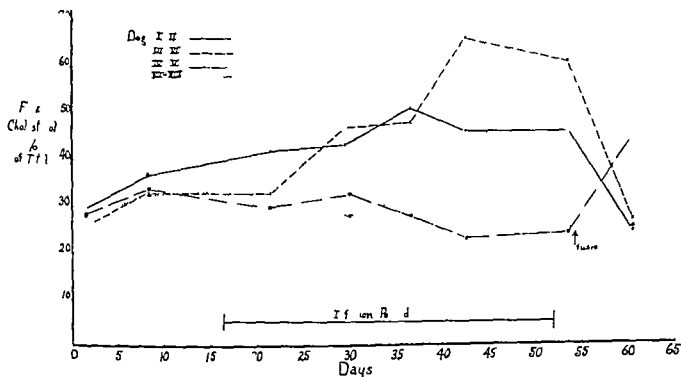


Fig 11—Cholesterol variations in all dogs in Experiment. Dogs I and II infused with 30 per cent fat emulsion. Dogs III and VI infused with phosphatide stabilizer without added fat. Dogs IV, V, VII and VIII not infused.

IV and V which received four infusions of a poor batch of emulsion beginning on the fifty-second day of the experiment are of interest. A week after the last of these infusions the ratio of free to total cholesterol had risen from a normal range of 0.25-0.33, to 0.46. There was a small increase of total serum cholesterol, and there was a prompt increase of bromsulfalein retention. Fig 11 is a graphic representation of the cholesterol data.

Except in Dogs IV and V after they had received the unstable and toxic emulsion, the liver function, as determined by serum bromsulfalein tests, revealed no abnormality to correlate with the serum cholesterol. Plasma prothrombin determinations at the close of the infusion period were normal.

The possibility of an increased fecal excretion of fat by the animals receiving fat emulsions was considered and hence twenty-four hour stool specimens were analyzed for total lipids. Table IV illustrates the fecal lipid excretion of the animals for an average period. It is seen that fecal lipids are not increased during the infusion of fat. Urinary studies showed that the urine contained insignificant amounts of lipids.

TABLE IV FECAL LIPID EXCRETION DURING FAT INFUSION IN EXPERIMENT 2

DOG	INFUSION	AVERAGE 24 HOUR FECAL LIPID EXCRETION (GM)
I	Fat, 5 Gm /kg	3.53
II	Fat, 5 Gm /kg	
III	Phosphatide, 0.5 Gm /kg	2.71
VI	Phosphatide, 0.5 Gm /kg	
IV	0	1.65
V	0	
VII	0	4.06
VIII	0	

*Pathology of Animals in Experiment 2* The animals in Experiment 2 were sacrificed for post-mortem study as indicated in Table III. The gross appearance of the organs and tissues was normal. Table V indicates the organ weights at autopsy. These were not unusual except for the spleens of animals III and VI which were significantly enlarged. Sections of the tissues were preserved in 10 per cent formalin for histologic study and other sections of the lungs, heart muscle, liver, spleen, pancreas, and kidneys were removed and immediately frozen at  $-20^{\circ}\text{C}$  and stored at this temperature for lipid analyses.

TABLE V ORGAN WEIGHTS OF ANIMALS IN EXPERIMENT 2 AT AUTOPSY

DOG	MATERIAL INFUSED	INFUSION DURATION (DAYS)	INTERVAL AFTER INFUSION BEFORE AUTOPSY (DAYS)	LUNGS (GM)		HEART (GM)	LIVER (GM)	SPLEEN (GM)	PANCREAS (GM)	KIDNEYS (GM)	
				L	R					L	R
I	Fat emulsion	31	16	40	65	95	530	75	25	30	30
II	Fat emulsion	31	16	45	65	115	560	65	29	37	37
III	Phosphatide fraction BF2	31	16	18	45	65	380	80	20	25	25
VI	Phosphatide fraction BF2	31	16	42	62	100	500	85	36	42	37
VII	0	0	0	35	52	97	385	37	32	33	33
VIII	0	0	0	37	54	78	371	24	23	26	28

Microscopic examination of sections of the lungs of the infused animals showed them to be normal. Sections stained with sudan III revealed no unusual fat deposition. Sections of heart muscle were normal as were the sections of aorta. No intimal lipid deposits could be demonstrated. The thymus and lymph nodes were not unusual and the adrenals appeared normal.

The spleens and livers of all infused animals showed an increase in pigment which appeared as brownish-black granules in the sinusoids and Kupffer cells. This pigment was more prominent in animals III and IV which had received the phosphatide stabilizer alone than in the animals receiving the emulsion of fat. All the infused animals were found to have occasional small focal accumulations of mononuclear cells in the liver, these consisted of four to ten cells negative for lipid by sudan stain. There were no multinucleated giant cells. There was no evidence of abnormal fat deposition in the liver.



The kidneys were found to be normal in all animals. The marrow unfortunately, was not suitably fixed for adequate study.

The histologic changes following the infusions were thus of two types—both minimal. First infused animals showed an increase of pigment deposition in the phagocytic cells of the liver and sinusoids of the spleen and second seated small round cell infiltrations were found in the liver.

*Lipid Analysis of Organs From Animals in Experiment 2* The frozen tissues were thawed and two portions of each organ were dissected free of adherent tissues. One portion was weighed wet dried in an oven at 105° C for

TABLE VI LIPID CONTENT AND DISTRIBUTION IN THE VARIOUS ORGANS AFTER INFUSION  
(All values expressed as grams per cent of dry weight; all values are means of duplicate determinations on tissues from two animals.)

ORGAN	INFUSION	FAT EMULSION (30% COCONUT OIL)	PHOSPHATIDE FRACTION (3% BE-)	CONTROLS (NO INFUSION)
Liver	Total lipid	13.8	13.9	13.2
	Total fatty acid	7.69	8.06	5.05
	Phospholipid	9.14	8.59	7.80
	Neutral fat	5.58	3.41	2.46
	Total cholesterol	0.74	1.0	0.45
	Free cholesterol	0.66	0.83	0.30
	Ester cholesterol	0.08	0.17	0.15
Lung	Total lipid	15.1	14.7	17.4
	Total fatty acid	7.73	7.42	9.45
	Phospholipid	8.79	9.04	9.90
	Neutral fat	3.01	2.16	4.02
	Total cholesterol	2.02	2.14	1.85
	Free cholesterol	2.01	2.02	1.85
	Ester cholesterol	0.01	0.12	0.00
Spleen	Total lipid	10.1	6.70	10.6
	Total fatty acid	4.17	2.86	4.45
	Phospholipid	6.28	3.58	5.90
	Neutral fat	0.81	0.94	1.14
	Total cholesterol	1.50	1.29	1.70
	Free cholesterol	1.53	1.29	1.70
	Ester cholesterol	0.00	0.00	0.00
Heart	Total lipid	13.6	12.1	18.5
	Total fatty acid	7.95	6.59	11.1
	Phospholipid	9.35	8.59	9.20
	Neutral fat	2.20	1.80	5.99
	Total cholesterol	0.61	0.54	0.55
	Free cholesterol	0.52	0.54	0.45
	Ester cholesterol	0.09	0.00	0.10
Kidney	Total lipid	15.2	16.6	17.5
	Total fatty acid	8.78	8.18	10.4
	Phospholipid	11.1	11.7	12.7
	Neutral fat	2.59	1.50	3.69
	Total cholesterol	1.28	1.75	1.55
	Free cholesterol	1.28	1.63	1.50
	Ester cholesterol	0.00	0.12	0.05
Pancreas	Total lipid	13.6	12.9	15.2
	Total fatty acid	8.46	6.92	8.85
	Phospholipid	8.85	4.58	6.4
	Neutral fat	3.75	4.50	5.40
	Total cholesterol	0.90	1.02	1.10
	Free cholesterol	0.87	1.02	1.05
	Ester cholesterol	0.03	0.00	0.05

forty-eight hours to a constant weight, and the percentage dry matter calculated. Another similar portion was weighed and ground with three times its weight of anhydrous sodium sulfate until a fine, pink powder was obtained. This material was air-dried, transferred quantitatively to Soxhlet thimbles, and extracted twenty-four hours with redistilled, dry chloroform. The chloroform was removed and the residue dried in vacuo. The residual lipids were taken up in low-boiling petroleum ether and filtered through fat free paper into a 100 ml volumetric flask. The contents were then made to volume with ether and aliquots taken for gravimetric determination of total lipids. Additional aliquots were taken for determination of total and free cholesterol by the Schoenheimer-Sperry method<sup>10</sup>, of phospholipids and free fatty acids by the titrimetric method of Man and Gildea<sup>17</sup>, and for determination of total lipid phosphorus using the method of Youngberg<sup>18</sup>. The per cent of total lipids present as neutral fat was then calculated by difference. These data are summarized in Table VI. It is seen that the total lipid content of the six organs examined from each animal was not significantly different in the four infused animals or in the noninfused animals. The values obtained are similar to those reported by other workers<sup>19</sup> for normal dogs and confirm the histologic evidence which revealed no abnormal lipid deposits in the tissues.

The livers of the four infused animals showed an increase of total cholesterol over the controls. This increase is associated with an increase of the ratio between the free to total cholesterol components. This was the only tissue which showed an abnormality of cholesterol content. The changes were of the type already described in the plasma, the total cholesterol content is increased with a marked increase in the ratio of free to ester cholesterol. As noted, the plasma cholesterol ratios had returned to normal sixteen days after the last infusion.

#### DISCUSSION

The data obtained in these studies give additional support to findings from this laboratory that a properly prepared emulsion of triglycerides given intravenously is utilized for energy requirements. The use of litters of growing puppies has proved to be a sensitive indicator of these functions. It has also been shown that proper selection of phosphatide stabilizers and careful preparation will give a stable emulsion of fat which can readily be infused in amounts up to 5 Gm of fat per kilogram of body weight per day. This amount of fat represents 20 to 30 per cent of the caloric requirement of an actively growing animal. Microscopic and chemical examination of the tissues of infused animals revealed minimal cellular reaction in the livers which is transient and after a few weeks leaves no apparent anatomical damage.

Although two of the puppies in Experiment 1 showed minimal evidence of histologic changes which may be attributed to the emulsions infused when sacrificed two to three weeks after the termination of the infusions, these changes were of questionable significance. Certainly there was no evidence of irreversible tissue damage as the result of continued infusions of large amounts of emulsion.

Animal 906 was found to be free of lesions four to five months later and Dog 909 which had received over 3 kg of fat intravenously in a period of sixty three days showed no histologic evidence of damage when examined at the end of the infusion period. Furthermore histologic data corroborate metabolic data indicating that the infused fat was disposed of in an orderly fashion and was apparently converted to energy and growth requirements. Even when as much as 10 Gm of fat per kilogram per day was infused there was no evidence that fat was deposited in the liver or other organs to the detriment of function at these sites. Chemical analysis revealed a disturbance of cholesterol metabolism leading to an increase of free cholesterol with a concomitant reduction of ester cholesterol in the plasma. A similar change of the cholesterol distribution and content was found in the livers of all infused animals sixteen days after the last infusion. There was no evidence of excessive deposition of lipids in the liver or other visceral organs. Liver function tests did not indicate injury to this organ other than the disturbance of cholesterol metabolism. A moderate anemia was consistently observed in the infused animals but it seemed to be self limited and improvement was prompt with cessation of the infusions. Since the anemia was of equal degree in the animals receiving only the phosphatide stabilizer it appeared not to be due directly to the fat. In many respects this anemia resembled the anemia of infection.

The relationship of appetite to the amount of infused fat suggests that the oral intake is determined in part by the total caloric requirement. The parenteral use of fat emulsions of high caloric concentration allows a promising approach to the problem of supplying adequate calories in a small fluid volume for individuals who are in caloric deficit and unable to take sufficient calories by mouth. However, if calories provided by vein are sufficient to reduce markedly the appetite and hence oral intake one may soon encounter or aggravate complications related to an inadequate intake of protein vitamins, or minerals unless care is taken to prevent the onset of such deficiencies.

The consistent observations that the immediate symptoms the anemia the transient liver lesions, and the disturbances of cholesterol metabolism were all as well marked in the animals receiving the phosphatide stabilizer alone as in the animals infused with fat emulsions suggest that despite efforts to improve and purify the phosphatide stabilizer this component of the emulsion was principally responsible for any undesirable effects that were produced.

#### SUMMARY

- 1 Two litters of puppies were used to assay the contribution of an intravenous emulsion of 30 per cent coconut oil to energy requirements
- 2 As judged by growth and nitrogen balance growing puppies were able to utilize up to 30 per cent of their total energy requirements supplied as fat emulsion intravenously
- 3 Chemical data indicated no disturbance of plasma proteins nonprotein nitrogen, bilirubin or liver function during the infusions
- 4 A moderate normocytic anemia developed in the animals that were infused, regardless of whether they were infused with fat emulsion or just

the phosphatide stabilizer. The anemia was self-limited, responded favorably when the infusions were stopped, and in many respects resembled the anemia of infection.

5 Histologic examination of the various visceral organs revealed no abnormal lipid retention. Occasional transient, focal collections of mononuclear cells were seen in the liver for a few weeks after the infusions.

6 Chemical lipid analysis revealed no lipid retention but did indicate a disturbance of cholesterol metabolism, both in the plasma during infusion and in the livers of infused animals.

We wish to express appreciation to the following companies who have supplied us generously with various materials used in this research: Associated Concentrates, Inc., Elmhurst, Long Island, N. Y.; The Upjohn Company, Kalamazoo, Mich.; Merck and Company, Inc., Rahway, N. J.; Research and Development Board of General Foods Corp., Hoboken, N. J.; Wilson Laboratories, Chicago, Ill.; Sheffield Firms Co., Inc., New York, N. Y.; Corn Industries Research Foundation, New York, N. Y.; and Anheuser-Busch Co., St. Louis, Mo.

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## THE ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS OF THE BLOOD DISCRASIAS

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REPORTS of the electrophoretic analysis of serum proteins in the several diseases involving the blood cells or their precursors are uncommon in the medical literature. In most instances the data presented have been recorded incidental to broader considerations of protein analysis or secondary to hematologic and histopathologic studies. Numerous reports have been made of the serum protein changes in multiple myeloma and characteristic patterns have been recognized.<sup>1, 2, 3, 4</sup> However, none of these have considered the effect on the serum proteins of a recently suggested form of chemotherapy. In view of the close relationship of diseases of the reticulo endothelial system<sup>5</sup> and the probable sites of formation and alteration of the serum proteins further investigation of the serum protein architecture of these related states appears indicated. In the present study the changes occurring by electrophoretic analysis of the serum proteins in various types of leucemia, pernicious anemia, infectious mononucleosis, polycythemia vera, and reticulum cell sarcoma are analyzed.

The object of this study was to learn (1) whether there is a pathognomonic serum protein architecture in one or more of the disease states under consideration and (2) if there is any correlation between the qualitative and quantitative changes observed, the related histopathology and the simpler clinical laboratory procedures.

### MATERIALS

The patients used for this study were admitted to the University Hospital between Sept 1, 1946, and Dec 30, 1947. The diagnosis was established by history, physical examination, and blood and bone marrow studies using the supravital and standard Wright's staining techniques. Routine and indicated special laboratory procedures including lymph node biopsy were accomplished in each instance.

The distribution of patients into diagnostic categories was as follows: chronic myelocytic leucemia three, acute myelocytic leucemia two, chronic monocytic leucemia two, acute monocytic leucemia four, chronic lymphocytic leucemia two, acute lymphocytic leucemia one, multiple myeloma four, reticulum cell sarcoma one, polycythemia vera one, infectious mononucleosis two, pernicious anemia in relapse two. The classification of patients and the acuteness or chronicity of a given case were determined by the cellular morphology of the peripheral blood and bone marrow in addition to the duration of illness prior to hospitalization.

### METHODS

All the serum analyzed was collected under sterile precautions while the patients were in the fasting state, prior to transfusion and all but symptomatic therapy. The exception to this rule was the second sample taken from patients with myeloma who had been treated

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Electrophoretic analyses



with Stilbamidine After collection the serum was refrigerated at  $-20^{\circ}\text{C}$  and the analysis was made after an interval of three to twenty one days had elapsed Aseptic technique was employed up to the time the serum was dialyzed preparatory to electrophoresis

The frozen samples were thawed and diluted from 7 volumes to 25 volumes with 0.10 ionic strength barbiturate buffer at pH 8.6 The diluted serum was then dialyzed at a temperature of  $1^{\circ}\text{C}$  against eight to ten times its volume of buffer thrice changed during a 72 to 168 hour period The dialyzed samples were then examined in a standard Klett electrophoresis apparatus using Longworth's technique<sup>7</sup> A 2 cc cell was employed for intervals up to 90 minutes at potential gradients of about 5 volts per centimeter at thermostat temperatures of about  $10^{\circ}\text{C}$  Several photographic exposures of each run were made The conductivity readings for the descending mobilities were made on the sample while those for the ascending mobilities were made on the buffer at  $0^{\circ}\text{C}$

Mobilities were determined from three different plates and averaged for each ascending and each descending boundary These two figures were then averaged to give the figure reported Concentrations were estimated by making enlargements from the photographic plates so that the new areas were from five to forty times the areas on the plates The resulting areas were then traced with a planimeter four times and the results averaged This procedure was done once for each ascending and descending component The values were then converted to per cent protein using  $\Delta n = 0.0200/\text{per cent}$  for albumin and  $\Delta n = 0.0219/\text{per cent}$  for all globulins Unless otherwise indicated the results given are the average of the values from the ascending and descending boundaries

#### RESULTS

The data from the electrophoretic patterns of the sera of the various disease entities are set forth in Table I Two of the normal analyses determined by us are added as well as the normal limits of mobilities approximate relative and absolute concentrations of the various fractions<sup>8</sup>

In the group of chronic leucemias listed there is a qualitative deviation from normal in the gamma globulin boundary which migrated at a slower rate than would be anticipated normally This phenomenon was observed in all types of chronic leucemia and was present in the sera from the patient with polycythemia vera and the one with reticulum cell sarcoma Generally the leucemic sera showed a decrease in the approximate absolute concentration of albumin The two cases of chronic monocytic leucemia and one of the acute cases had normal albumin values The decrease in albumin in leucemia was suggested by Keilhack<sup>9</sup> using the Howe technique of sodium sulfate precipitation One of the patients with chronic monocytic leucemia (3a) was seen early in the disease and at that time had a high normal albumin concentration As the disease progressed this value became markedly lower

The leucemic sera rather uniformly demonstrated a rise in the approximate absolute and relative concentration of alpha 1 and alpha 2 globulin The cases of reticulum cell sarcoma and polycythemia were slightly elevated in this moiety These globulins were within normal limits in the sera of the cases of pernicious anemia and infectious mononucleosis

The leucemic sera in general (except the chronic lymphatic) demonstrated a rise of the approximate absolute and relative concentration of beta and gamma globulin The sera of the chronic lymphocytic leucemias were below normal for gamma globulin Both cases of infectious mononucleosis demonstrated a rise in the relative and absolute values for gamma globulin Sera from the patient

with reticulum cell sarcoma showed a marked rise of gamma globulin both relative and absolute, and normal beta globulin. The case of polycythemia presented low values for beta and gamma globulin. The early case of chronic monocytic leucemia and the acute monocytic leucemias demonstrate a rather marked increase in the relative gamma globulins. The cases of pernicious anemia were within normal limits for the approximate relative and absolute values of beta and gamma globulin. The total protein concentration values of all sera

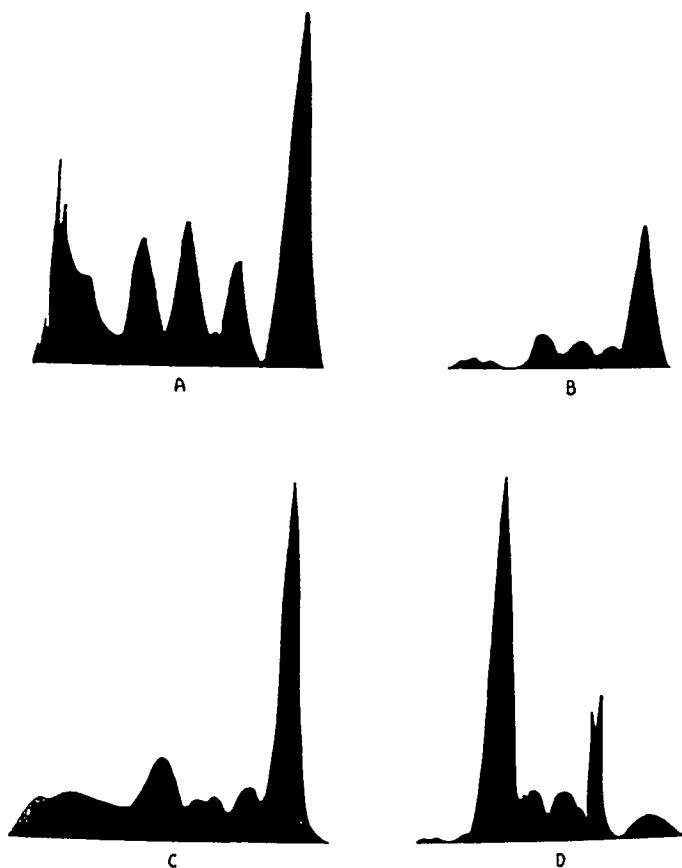


Fig 1

fractionated were not comparable with those determined by the routine clinical methods, and in all but one sample the albumin-globulin ratio was lower when computed on the basis of the electrophoretic analysis. These albumin globulin ratios determined by electrophoresis fall well below the accepted normal values.<sup>10</sup>

Representative schlieren patterns are shown in Fig 1. Sera from one of the patients with acute monocytic leucemia demonstrated a small spike on the gamma globulin peak which we have not observed in normal subjects and which was seen in previous analyses of this type of sera (Fig 1, A). Fig 1, B is a pattern from a patient with chronic lymphocytic leucemia which displays a very low gamma globulin peak. One pattern (Fig 1, C) from a case of monocytic leucemia shows a notching of the alpha-2 peak which was present but not



TABLE II

CASE	MOBILITY					ABSOLUTE CONCENTRATION					RELATIVE CONCENTRATION					STIL BAMIDINE	MAPPW
	ALB	$\alpha$	$\alpha$	$\beta$	$\gamma$	ALB	$\alpha$	$\alpha$	$\beta$	$\gamma$	TP	$\alpha/\alpha$	$\beta/\alpha$	$\gamma/\alpha$	$\Delta/c$		
1	5.99	5.00	3.85	2.82	1.04	1.47	26	32	5.61*	22	8.10	17	22	3.13*	16	27	48% plasma cells
2 A	6.29		3.88	2.82	85	3.68	50	98	1.06†	7.20	13.24	14	26	32	2.04	39	84% plasma cells
B	6.16	5.20	4.04	2.92	87	3.61	60	69	1.20	10.07	15.00	17	20	32.2†	24		71% plasma cells
3, A	6.00	5.00	3.68	2.12	90	2.31	36	87	6.63	12		17†	31†		38	3300 mg	37% plasma cells
B	5.88	4.97	3.84	2.62	115	3.40	31	38	12.74	1.34	15.10	09	11		37	22	25% plasma cells
4 A	6.33	5.47	3.94	3.17	77	4.79	56	16	8.9†	14.52	20.92	12	34		03	3300 mg	40% plasma cells
B	5.64	4.67	3.60	2.55	116	4.26	32	54	1.00	11.43	17.21	09	12	24	11	39	36% plasma cells

\*Descending only

†Ascending only

A Before treatment

B After treatment

as marked in chronic myelocytic leucemia. Fig 1, *D* is from a case of chronic myelocytic leucemia and discloses an unusual abnormality of the beta globulin spike.

The data from sera of patients having a confirmed diagnosis of multiple myeloma are presented in Table II. The architecture before treatment and in the untreated cases confirms the report by Kekwich<sup>1</sup> that there are two types of abnormal patterns in myeloma, a high beta globulin peak and a high gamma globulin peak. There seems to be no obvious correlation that can be made regarding any quantitative or qualitative changes in the peripheral blood protein pattern after the administration of Stilbamidine. The values are shown in Table II.

#### DISCUSSION

In a study of the changes in mobility of the electrophoretic pattern of serum following experimental burns, Peilmann and co-workers<sup>11</sup> found a new boundary which migrated at a slower rate than gamma globulin. Seibert<sup>12</sup> noted a similar component in half of the tuberculosis cases analyzed. One analysis of a patient with acute lymphocytic leucemia reported by Longworth and associates<sup>13</sup> shows a tendency to slow mobility at the gamma globulin boundary. Though our cases of chronic leucemias showed this tendency to slowing, none of them displayed a separation of the peak into two parts.

The generally lowered absolute concentration of albumin has been noted in various pathologic states. Leutscher,<sup>14</sup> in his review of the subject of electrophoresis points out the common occurrence of this trend and hence it cannot be considered as characteristic of the blood dyscrasias. This deviation from normal has been pointed out by other authors<sup>9-13</sup> in both acute lymphocytic and myelocytic leucemias.

The absolute concentrations of the alpha-1 and alpha-2 globulins were generally increased over normal in the leucemias of this study. It has been stated that an increase in the alpha globulin is the first change which takes place in acute injury or infection.<sup>14</sup> The fraction of alpha globulin which is responsible for this increase was not designated. However, it was present in the chronic as well as the acute leucemias of this series. The relative values of alpha-1 and alpha-2 globulin were similarly increased. Chow<sup>15</sup> has shown that the rise of alpha globulin may be dependent on a fall of albumin, with normal values for the alpha globulin attained by increased protein intake and restitution of the albumin fraction. However, in several of our cases (Table I, 3,b, 4,c) there were elevated absolute alpha globulins, the albumin fraction being within normal limits. Furthermore, in one case of chronic lymphocytic leucemia the administration of 50 Gm of serum albumin daily for a period of fourteen days restored the albumin to normal levels and only slightly altered the alpha 1 globulin. However, the alpha-2 globulin approximated normal and the gamma globulin content remained essentially the same. In one case of chronic myelocytic leucemia, placing the patient on a high protein diet supplemented with protein hydrolysates did not restore the albumin fraction or alter the alpha globulins appreciably even though all liver function studies were within normal limits.

A relative elevation of the gamma globulin is said to be a phenomena observed in the chronic state of disease and injury<sup>14</sup>. Our data would tend to support this, though the aberration was prominent in some acute cases and absent in a few of the chronic ones.

Both cases of chronic lymphocytic leucemia present a low value for both relative and absolute gamma globulin. Howell<sup>17</sup> noted that leucemic individuals do not respond normally to infection or to injected antigens. Krebs<sup>18</sup> noted diminished value of gamma globulin in simple malnutrition. One patient with chronic lymphocytic leucemia given a series of injections intravenously of typhoid vaccine did not develop antibodies as shown by the Widal test which remained negative. The administration of extract of the adrenal cortex did not alter the antibody response or the electrophoretic pattern. Repetition of this experiment after restitution of the albumin fraction did not influence the gamma globulin. Blood volume determinations<sup>19</sup> prior to and following the administration of extract of the adrenal cortex demonstrated a moderate increase and this factor was taken into account in the latter determination. It is possible however that the pituitary adrenotrophic hormone may be involved in the gamma globulin component as Dougherty and White have pointed out in individuals with normal lymphocytes.<sup>20</sup>

In the cases of monocytic leucemia and reticulum cell sarcoma, marked elevation of absolute and relative gamma globulins is seen. The explanation for this rise is obscure but may be related to the similarity of the fundamental histopathologic defect in both entities, the reticulum cell and the monoblast being closely related in the polyphyletic tree.<sup>1</sup>

Both patients with infectious mononucleosis had elevated absolute and relative gamma globulin values and moderate diminution of the albumin fraction. The heterophile antibody was strongly positive in both cases. Although the etiology of this disease is unknown,<sup>22</sup> much evidence has been submitted<sup>23</sup> demonstrating the widespread involvement of the reticulo endothelial system and other organ systems which could account for the deviation of the protein pattern from normal. The cases of pernicious anemia in relapse both demonstrated normal albumin values and normal globulin fractions. As would be expected from the approximate relative or absolute increase of the various globulin fractions, the albumin globulin ratio was consistently decreased, often to subnormal levels and even less than 1.0. All were below the range within which 68 per cent of the normal cases should fall and most were not within the limits which includes 94.5 per cent of normal subjects.<sup>9</sup>

In Table III there is a comparison of the electrophoretic protein determinations and the results of the clinical laboratory procedures performed in the respective disease entities. The routine laboratory protein determinations were not comparable to the total protein or A/G ratios as determined electrophoretically. The latter technique in most instances gave uniformly lower ratios than those obtained by the routine clinical laboratory methods. Nitske and Cohen<sup>24</sup> recently have demonstrated the inadequacy of the routine total protein determinations in cases of Hodgkin's disease and leucemia. These authors utilizing a methyl alcohol fractionation technique, demonstrated lower albumin and elevated globulins by this method than by the usual clinical methods.

TABLE III

DISEASE	TOTAL PROTEIN		ELECTROPHORETIC TOTAL PROTEIN		BUN	BMR	THIN MOL	CEPH FIOC	H <sub>2</sub> O THINOM BIN	ACID PHOS TASE	WBC	ABSOLUTE IMMATURE	VPIOW Myeloid hyperplasia "B" level Blast cells 6% Myeloid hyperplasia Mature level Blasts 2% Myeloid hyperplasia "C" level Blasts 5% Myeloid hyperplasia Blasts 55% Myeloid hyperplasia Blasts 42% Monocytes 39% Monoblasts 6% Monocytes 31% Monoblasts 8% Monocytes 4% Monoblasts 31% Monocytes 5% Monoblasts 11% Monocytes 1% Monoblasts 17% Monocytes 20% Monoblasts 18%
	6.66	2.74	6.32	A/G 1.12	21.5	+49	20.0	+	30.5	5.4	742,000	497,000	
Chronic myelocytic	6.94	1.81	6.16	1.30	11.5	+22	0.0	-	12.0	4.68	206,000	127,000	
	6.35	2.68	6.50	1.00	18.0	+46	0.0	+	59.0	6.4	158,000	207,000	
Acute myelocytic	5.71	1.65	5.23	.68	7.5	+36	20.0	+	54.0	7.6	48,000	26,400	
	5.94	1.45	4.87	1.35	12.0	+32	10.0	+	60.0	3.8	76,000	31,920	
Chronic monocytic	8.68	1.00	7.64	1.28	13.5					3.7	24,000	2,880	
	8.52	.98	9.50	1.08	10.6	+18	10.0	-	114.0	3.5	1,350	68	
Acute monocytic	6.66	1.51	4.89	.63	20.0		40.0	+	48.8		500	135	
	5.94	1.45	4.87	.64	17.0	+36		+	53.6		1,550	62	
	6.25	1.47	8.88	1.37	11.5		20.0				1,800	144	
	7.48	1.22	8.79	.74	15.5		10.0	±			2,250	315	

Chronic lymphocytic	5.78	4.78	4.15	1.14	14.0	+43		-		51	320,000	0	Lymphatic infiltration 95%
	5.74	4.4	4.35	1.54	16.2	+30		+	55.0	34	294,000	0	Lymphatic infiltration 75%
Acute lymphocytic	5.4	2.03	4.35	87	33.5	+60	10.0	±	69.0	46	66,000	61,380	Lymphatic infiltration 90%
	7.06	1.00	5.59	60	14.0	+38	40.0	++++	33.0	354	5100	0	Sarcoma cells 32%
Polycythemia rubra vera			3.62	1.63	14.5	+20	0.0	++	-	420	9,000	16,560	Generalized hyperplasia all elements
	6.98	1.74	6.55	1.0	13.2	-4	5.0	++	92.0	30	4500	3600*	Not done
Pernicious anemia relapse	8.37	1.44	7.08	1.13	10.0	+5	10.0	+++	100.0	32	13,000	8,315*	Heterophile 1.7168 Rieder cells 4%
	4.74	4.40	6.4	1.87	4.0		10.0	±	32.0	44	10,900	0	Heterophile 1.14336 Megaloblast hyperplasia 32
	3.44	1.86	7.46	1.65	16.0		10.0	+	61.0	35	3,900	0	Megaloblast hyperplasia 26

\*Rieder cells.

The correlation of the changes in protein architecture and the varied clinical laboratory procedures are particularly difficult to evaluate as the primary disease process, and many secondary effects of the disease may alter each determination. The albumin-globulin ratio or total protein determination did not bear any consistent relationship to the other procedures. The basal metabolic rate and the uric acid determinations were more consistently involved or altered in the leucemic state. These two determinations have been repeatedly investigated in this condition and are assumed to be elevated by increased protein catabolism and the breakdown of leucocytes.<sup>20</sup> The cephalin flocculation and thymol turbidity tests were markedly elevated and the prothrombin time diminished when the excretory tests of liver function showed alteration. The thymol turbidity and cephalin flocculation tests in low titers bore no constant relationship to an abnormal A/G ratio and their aberrations may be explained in part by the occurrence of abnormal amounts of beta and gamma globulin in the sera. Cohen<sup>6</sup> has demonstrated by electrophoretic studies the relationship between the thymol turbidity test and the beta globulins. The cephalin flocculation test is also reported by this author to be more closely parallel to increases of gamma globulin.

The degree of infiltration of the bone marrow by leucemic cells was determined by performing four differential counts of 200 myeloid cells each and averaging the results. By this gross determination the degree of infiltration appeared to reflect itself in a lower A/G ratio by the electrophoretic technique. No correlation between the small numbers of plasma cells in the bone marrow, lymph nodes, or autopsy specimens and the protein architecture was noted.

Investigation of myeloma serum by electrophoresis has been as thorough as that of any single disease, and its schlieren patterns can be as nearly pathognomonic as those seen in any morbid state. As stated, our results confirm those in the literature which refer to two abnormal types of myeloma patterns, one with a marked increase in the area of the beta globulin (cases 1 and 3) and the other with the major protein increase in the gamma globulin fraction (cases 2 and 4). In treated patients, the one with the beta elevation prior to treatment showed an increase in this fraction following therapy. In the gamma type no consistent effect of Stilbamidine on the protein architecture was observed. No significant changes were noted in the peripheral blood or bone marrow that could be ascribed to the therapy.

#### SUMMARY

The electrophoretic patterns of various blood dyscrasias are presented. The leucemic states are associated with a diminution in the approximate absolute amount of albumin and a rise in the absolute amount of globulin. The albumin-globulin ratios fall below the limits of normal in most instances. The alpha-1 and alpha-2 globulin are increased in most instances and the increase is noted with both normal and diminished total albumin values. Gamma globulin values, both absolute and relative, were elevated in monocytic leukemia, reticulum cell sarcoma, and infectious mononucleosis. Chronic lymphocytic leukemia demonstrated low relative and absolute gamma globulin values. A markedly lowered albumin-globulin ratio appears related to the degree of infiltration of the bone

marrow by leucemic cells as well as when the excretory and metabolic functions of the liver demonstrate impairment. No alteration in the serum protein architecture was noted following Stilbamidine therapy.

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# ELECTROLYTE PARTITION IN PATIENTS WITH EDEMA OF VARIOUS ORIGINS

## QUALITATIVE AND QUANTITATIVE DEFINITION OF CATIONS AND ANIONS IN CARDIAC DECOMPENSATION

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THE role of the kidneys in the production of edema has been examined by various groups of investigators<sup>1-5</sup> as methods for the study of renal physiology have been developed. Although agreement has not been general concerning the sequence of events transpiring in the kidneys during the accumulation in the tissues of edema fluid, it is at least conceded that such events do transpire and that salt and water are not simply diffusing across the capillary membrane in response to an elevation of hydrostatic pressure, but are being incompletely excreted by the kidney. This concept is by no means novel, and appears in standard texts of a decade or two ago.<sup>6</sup> Further clarification, however, has been delayed by the difficulties, inherent in the study of renal physiology in man, of establishing values for filtration rate sufficiently accurate to serve as a basis for the calculation of reabsorbed substances. The volume of plasma worked by the kidneys in a given period of time is so large, and the defect in salt and water excretion capable of leading to edema is relatively so small, that the significant increment tends to be lost in the physiologic variations of the inulin or mannitol clearances as we are able to determine them at the bedside. Thus, if the clearance of inulin is found to vary only to the extent of plus or minus 5 or 10 c.c. per minute, that range of error, or physiologic variation, is so large in relation to the few cubic centimeters of water or fractions of milliequivalents of sodium which represent the retention characteristic of cardiac decompensation, that physiology described in these terms must be accepted with reserves. Even more inaccessible to the clinical investigator are determinations capable of yielding conclusive information concerning the factors governing diffusion and active reabsorption in proximal and distal tubules and the stimuli which may modify these processes in disease states.

Bearing in mind these limitations, we have chosen to review in detail the alterations in urine electrolytes earlier reported in group studies<sup>7</sup> confined to sodium and chloride. The depression of urinary sodium chloride ratio in cardiac failure appeared to be a constant finding implying a higher degree of specific selective tubular reabsorption than has been generally recognized. Through a more complete delineation of relationships, with varying electrolyte

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loads it was our purpose to define the variation of electrolyte patterns and fluid balance with sufficient accuracy and completeness as to bring out characteristics tending toward a recognition of possible causal agents. In adopting an approach similar to that of Gamble we were able to avail ourselves of accepted data pertaining to the normal state.

#### METHODS

The two subjects selected for this study were suffering from rheumatic heart disease with congestive failure. F. W. was a 36 year old woman with mitral stenosis and insufficiency who was hospitalized in congestive failure. She was discharged improved after the experimental period but was twice rehospitalized during the subsequent six months because of fluid retention. W. S. was a 28 year old man who was first seen in the clinic and hospitalized because of advanced rheumatic heart disease with congestive failure. He was discharged improved after the reported period of study and treatment but was later admitted to a veterans hospital in terminal condition. The autopsy report indicated rheumatic involvement of the aortic and mitral valves, cardiac cirrhosis, and pulmonary infarction.

Both patients were kept in bed rest for the first fortnight of their hospital stay after which they were permitted to move about the wards as they wished. Both were allowed fluids ad libitum and both were given a low salt acid ash diet. The estimated electrolyte content of the diet, in millimols per day, was

Sodium	17.61 (average 44)
Potassium	103
Calcium	13
Magnesium	12
Iron	4
Phosphorus	63
Sulfur	32
Chloride	13.46 (average 30)

Urine specimens were collected under toluene and were kept refrigerated throughout the collection period. pH was determined on the samples as soon as they were received in the laboratory. If ammonium and phosphate determinations could not be carried out immediately aliquot samples were frozen in solid carbon dioxide and were maintained at  $-10^{\circ}$  in a Dewar flask until ready for use.

Analytic procedures used in the determination of ionic constituents were for the most part as recommended by Peters and Van Slyke.<sup>8</sup> All determinations were performed in duplicate or triplicate.

**Sodium**—Gravimetric method of Butler and Tutthill. Some of the gravimetric urinary sodium values were checked using a colorimetric adaptation of this procedure.

**Potassium**—Gravimetric procedure of Freeman and Burrill.<sup>9</sup>

**Calcium**—Microtitration by the method of Tisdall and Kramer.

**Magnesium**—Precipitation according to the method of Simonsen, Westover and Wertman.<sup>10</sup> Color development however, was by the phosphomolybdate technique of Gomori.<sup>11</sup>

**Ammonia**—Determination was by nesslerization following aeration from alkaline solution as in the method of Folin and Farmer.<sup>1</sup>

**Chloride**—Modified Volhard-Harvey titration. In many instances especially when protein was present these were checked by the Van Slyke-Hiller<sup>12</sup> modification of Sendroy's colorimetric procedure.

**Phosphate**—The method of Gomori<sup>11</sup> was used after decolorization and dilution. Milligrams phosphorus were converted to milliequivalents phosphate ( $\text{HPO}_4$ ) by use of the appropriate factor as indicated by the pH of the sample.

**Inorganic Sulfate**—Turbidimetric measurement of the barium sulfate precipitate.<sup>14</sup> This is admittedly a comparatively crude method but it gave satisfactory results and recoveries.

Recently reported deceased at home.



**Organic Acids**—Total twenty four hour excretion was calculated from aliquots titrated according to Van Slyke and Palmer. Creatine and creatinine were removed by shaking with Lloyd's reagent as recommended by Greenwald<sup>15</sup>. Titration was carried out between pH 8.0 and 2.7, using a glass electrode for determination of end points. Correction for the existence of some of the acid in the free form as recommended by Gamble<sup>7</sup> was not made because titration curves indicated such a multiplicity of constituents that it was impossible to estimate which was the predominant acid.

Filtration rates and effective renal plasma flow were determined by the clearances of mannitol and sodium para amino hippurate. Analytic procedures were those of Corcoran and Page<sup>16</sup> and H. W. Smith and associates<sup>17</sup>.

Serum electrolyte determinations were made by adaptations of the methods already described for urine.

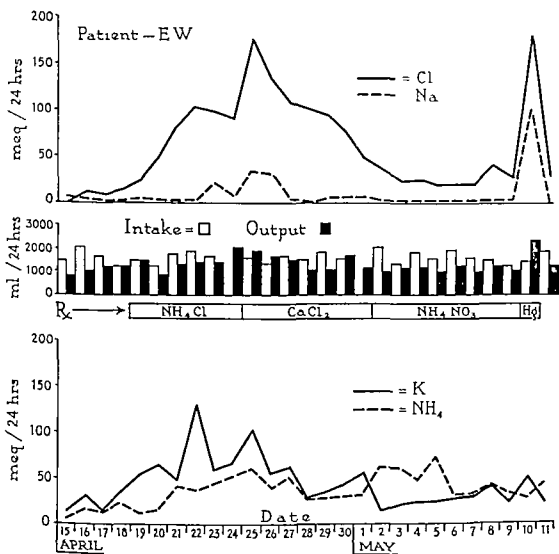


Fig 1—E. W. electrolyte and water excretion with diuretics

## RESULTS

During control periods as throughout the administration of diuretics, sodium was consistently low both in relation to intake, total ionic concentration and to chloride.

Other electrolytes showed no significant variation. E. W. maintained a consistently higher level of calcium output than did W. S., a finding consistent with previous observations that individual levels tend to be constant though variable from subject to subject.<sup>18</sup>

Of the mineral diuretics administered, ammonium chloride, potassium chloride, and potassium acetate appeared to be most effective as diuretic agents. It is notable that the maximum effect of each of these substances was reached around the third day of therapy, after which both fluid and electrolyte values declined toward control levels. For example (Fig 2), on May 23 the urine volume had risen from 1,590 to 2,230 cc with an almost identical intake, and the sodium and chloride, respectively, from 5 to 75 meq and 34 to 160 meq. After this diuretic peak, the output of salt and water declined notwithstanding the continuation of the same medication.

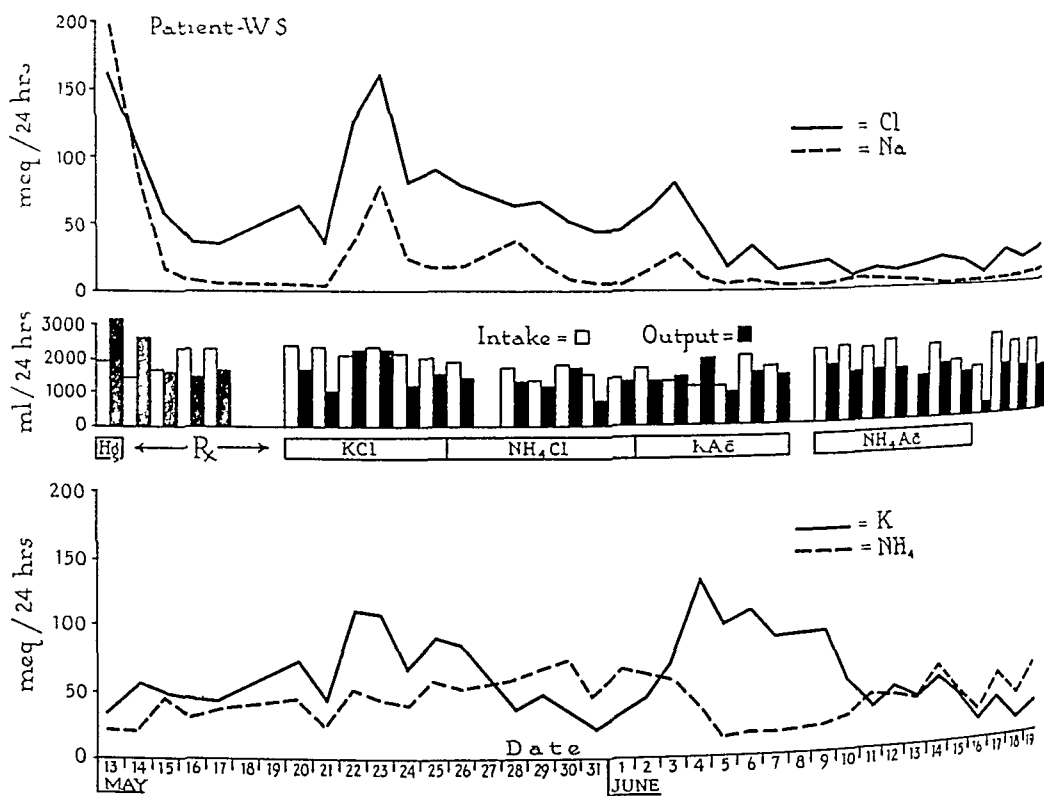


Fig 2—W S electrolyte and water excretion with diuretics.

Although minor responses in the sodium output were found with ammonium chloride, potassium chloride, and potassium acetate (Figs 1 and 2), the daily output tended to remain at a level far below normal values on varying salt intakes.<sup>5, 10</sup>

Certain findings peculiar to the specific diuretics are notable. Calcium excretion is distinctly increased with ammonium chloride, thus W S (Table II) on May 30 showed a calcium value of 11.3 meq, with 5.9 meq before the beginning of the drug. Potassium acetate was effective in increasing water output, but the urine resulting from its administration was poor in sodium and chloride. As previously reported,<sup>10</sup> a prompt and sustained alkalinizing effect was demonstrated.

TABLE II. URINE ELECTROLYTES (IN MEQ/24 HOURS) PATIENT W. S.

DATE 194	INTAKE	B	OUTPUT	pH	Na	K	NH	Ca <sup>++</sup>	Mg <sup>++</sup>	Cl	UPO	INOR GANIC SO	OR GANIC ACIDS	WT
May 10	(Admitted to hospital)													127½
May 11	Mercurpurin 2 ml		3250	7.0	197	37	22	4.4	6.1	159	59	11	30	122½
May 13	1800		2600	6.2	90	56	21	3.3	6.6	106	39	15	40	118
May 14	1400	No medication	1570	5.5	16	48	44	2.0	3.8	58	24	17	25	115
May 15	1700	No medication	1400	5.6	9	45	32	2.4	3.4	36	17	16	22	114
May 16	2300	No medication	1590	5.6	5	44	36	2.4	3.7	34	20	19	24	114
May 17	250	No medication	1650	6.0	4	74	45	5.8	6.3	63	26	25	25	115
May 20	2300	8 Gm KCl	1010	5.6	3	44	24	2.8	3.1	124	24	25	22	115
May 21	2300	8 Gm KCl	2220	5.8	30	111	50	5.2	5.0	124	24	25	22	115
May 22	2100	8 Gm KCl	2230	5.6	75	108	45	7.0	3.7	160	24	30	35	115½
May 23	2330	8 Gm KCl	1150	5.7	23	68	40	3.4	2.7	89	23	25	24	115½
May 24	2100	8 Gm KCl	150	5.2	18	89	57	5.9	3.1	89	23	25	24	115½
May 25	1990	8 Gm KCl	1370	5.4	17	84	53	5.7	2.5	78	22	26	27	116
May 26	1900	12 Gm NH <sub>4</sub> Cl	1240	5.4	17	84	53	5.7	2.5	78	22	26	27	116
May 28	1700	12 Gm NH <sub>4</sub> Cl	1240	5.4	17	84	53	5.7	2.5	78	22	26	27	116
May 29	1300	12 Gm NH <sub>4</sub> Cl	1060	5.0	20	48	67	10.6	4.5	66	19	17	24	115
May 30	1800	12 Gm NH <sub>4</sub> Cl	1680	5.4	9	34	72	11.3	4.0	52	19	27	27	115
May 31	1500	12 Gm NH <sub>4</sub> Cl	665	5.4	5	23	47	8.0	2.6	43	15	14	20	-
June 1	1400	10 Gm potassium acetate	1290	5.5	4	45	66	7.9	3.5	46	15	21	21	117
June 2	1600	10 Gm potassium acetate	1240	5.3	15	45	62	8.7	2.2	60	15	22	27	115½
June 3	1300	10 Gm potassium acetate	1200	5.3	26	71	50	6.4	3.5	84	16	25	27	116½
June 4	1200	10 Gm potassium acetate	1940	7.0	9	132	39	4.1	2.2	46	29	34	32	116
June 5	1130	10 Gm potassium acetate	890	8.0	5	99	14	2.5	2.2	16	17	19	22	116½
June 6	2100	10 Gm potassium acetate	1530	7.1	6	109	17	2.9	2.2	31	29	31	30	117½
June 7	1700	10 Gm potassium acetate	1410	7.3	3	90	16	3.1	2.4	13	24	18	24	118
June 9	2180	8 Gm ammonium acetate	1640	6.9	3	92	19	3.7	2.8	11	29	21	24	118½
June 10	200	8 Gm ammonium acetate	1420	5.9	7	52	23	3.5	2.9	10	21	21	26	118½
June 11	2400	8 Gm ammonium acetate	1380	5.5	5	42	39	4.7	2.7	13	22	21	23	118½
June 12	2400	8 Gm ammonium acetate	1240	5.7	4	37	35	5.3	3.5	17	23	17	28	118
June 13	2200	8 Gm ammonium acetate	1610	5.8	3	49	38	6.8	3.3	22	21	22	35	119½
June 15	1740	8 Gm ammonium acetate	1320	5.6	3	37	39	3.4	1.5	18	17	17	28	118½
June 16	1490	No medication	310	5.7	1	10	3	2.4	1.1	8	23	10	13	118½
June 17	2450	No medication	1440	5.7	3	84	48	3.1	2.2	23	23	23	26	119½
June 18	2100	No medication	1360	5.6	4	14	33	3.2	1.9	19	13	14	23	119½
June 19	1500	No medication	1240	5.5	7	3	53	4.3	1.8	24	14	14	26	120

Mercurial diuresis in W S resulted in a sodium value of 197 meq, with a chloride of 159 meq, giving a ratio of 1.2. In the case of E W, the increase in sodium excretion was substantial but not sufficient to approach a normal ratio with the chloride. In one instance (W S) the discrepancy between sodium and chloride appeared to be compensated by a threefold increase in phosphate excretion. Other electrolytes were unaffected.

Filtration rates and effective renal plasma flow are given in Table III. The filtration rate in the case of E W was within normal limits before and throughout the period of this study. That of W S, was reduced to approximately 50 per cent of normal. This marked disparity in filtering power was accepted as a desirable qualification in the selection of these subjects.

TABLE III

PATIENT	FILTRATION RATE (ML/MIN)	EFFECTIVE RENAL PLASMA FLOW (ML/MIN)
E W	119*	534*
W S	56†	303†

\*Average of twelve determinations

†Average of three determinations

## DISCUSSION

The choice of total quantitation of individual electrolytes as an approach to the evaluation of the kidney in edema formation appeared to offer several manifest advantages. If, indeed, the impression of various workers is correct that the kidney is implicated in the events leading to fluid retention, possibly in a prime role, then it should be determinable whether such intervention derives from the process of filtration or of reabsorption, and whether it is autonomous or responsible to some other agent.

Assuming that the serum sodium values\* represent unbound sodium in these patients as in the normal, then the sodium filtered daily may be readily calculated and will be found to average 22,381 meq in the case of E W, and 9,438 meq in that of W S†. Comparison of these values with the approximate intake daily of 44 milliequivalents shows a disparity so impressive as to discourage the attempt to relate failure of excretion to failure of filtration since the latter function has with respect to the major tissue electrolytes such a wide margin of safety. The effective renal blood flow is seen to be within normal in E W and reduced by approximately 50 per cent in W S, it is evident that renal impairment as indicated by a reduced filtration rate and blood flow is not invariably associated with cardiac decompensation.

It is likewise noteworthy that impairment of the kidney would be expected to diminish renal working capacity. The demonstrated reabsorption of excess quantities of sodium, hence performance of increased osmotic work, indicates the reverse situation. It seems plausible to hypothesize, therefore

\*Plasma sodium and chloride concentrations for E W were 139 meq and 102 meq respectively. Those for W S at time of discharge were 119 meq and 107 meq. The low sodium level in the latter case may have been associated with the existing secondary cirrhosis.

†Filtration rate (ml/min)  $\times$  serum Na<sup>+</sup> (meq/ml)  $\times$  1440 = meq/24 hours

that the stimulus to the excessive reabsorption of sodium is specific and that it depends upon physiologic factors, chemical or humoral, rather than upon an injured or invalid kidney

The behavior of sodium with the administration of mineral diuretics as indicated in Tables I and II is predictable in terms of primary response.  $W/S$  is seen to increase urine output somewhat and concomitantly sodium output with potassium chloride, ammonium chloride, and potassium acetate. With the first two substances in particular, the sodium increase is many times greater than that of water, and, notwithstanding the administration of the chloride salt, the chloride output is less sharply increased than is the sodium. In no case did the diuretic effect last more than two to four days, although the medication was continued for several more days.

The brief duration of diuresis is an observation of clinical importance as well as physiologic interest. If potassium chloride for example, is capable of augmenting the urine volume for so short an interval of time it could well be argued that a rotation of mineral salts might be adopted in the management of chronic cardiac decompensation.

In both patients the administration of a mercurial diuretic resulted in a urine output of approximately twice that of the control levels, but the increase in sodium output was even more marked. The release of salt and water by mercurial preparations is by no means a novel observation, but the absence of significant changes in other electrolytes is a matter of considerable theoretic interest. If the augmented absorption of sodium and, to a lesser degree, chloride were a function of a factor so nonspecific as renal blood flow, it is difficult to conceive of that factor as being abolished by the administration of mercury to which a marked stimulation of renal circulation could hardly be attributed.<sup>21</sup>

Turning now to the excretion of potassium as shown in Tables I and II, only minor fluctuations are observed which with those of ammonia, probably reflect shifts in cation anion balance. A powerfully alkalinizing effect is noted in the urine under the influence of potassium acetate a result which serves a useful purpose in clinical practice where alkalinization by sodium salts might be undesirable. It is also of interest that when the potassium intake is elevated by the ingestion of 100 millimols of potassium salt a positive balance is established and a considerable portion of the element is held in the body.

The values for ammonia in the urine show the normal response to the ingestion of ammonium chloride. As in Grimble,<sup>7</sup> Chart 29, an elevation follows that of chloride and is prolonged beyond the chloride. A tendency to inverse proportionality with sodium and potassium can be traced compatible with the classical theory of ammonia as a spacer of fixed base. This interpretation becomes open to question in the study of hepatic cirrhosis presented in the following paper. Organic acids found in the urine underwent virtually no changes throughout the period of the experiments. The urine calcium, magnesium sulfate and phosphate offer no particular grounds for discussion within the framework of the present study.

The described shifts in electrolyte components should be considered as metabolic renal function tests. As such they reveal no suggestion of renal incompetence. The probability is strong, therefore, that a kidney which is able to maintain acid-base balance, preserve blood levels, and synthesize ammonia, is retaining sodium in response to factors acting upon it and not through incompetence.

Considering now the situation in terms of fluid compartments, the clinically visible increase in extracellular volume characteristic of cardiac decompensation is analogous to the situation described by Stewart and Rouike<sup>2</sup> in which large infusions of isotonic sodium chloride are given daily for four days and the extracellular fluid is thus increased by approximately 80 per cent of the original volume. Examining the data reported by these investigators, we find that at the end of three days the positive fluid balance has been reversed and the urine output exceeds the water intake by almost 1 liter. The sum of the excreted sodium and chloride liberally exceeds the intake after the first twelve-hour period, and the ratio of sodium to chloride in the urine never drops below 0.89. The plasma values are not extraordinary. If it is objected that the increased venous pressure present in cardiac decompensation could not have operated in Stewart's normal subject, it might be pointed out that this situation was well simulated by the forced increase in plasma volume resulting from the daily administration of 6 to 7 liters of 0.9 per cent sodium chloride. If we may then concede that the cases are comparable with respect to the fluid compartments, we are in a position to appreciate the disturbance in excretory water and electrolyte pattern revealed in the decompensated state. It appears evident either that the normal pituitary control of fluid volumes and electrolyte concentrations is defective in the cardiac patient or that the kidney is not able to respond normally to such control.

With respect to the second possibility, namely, tubular decompensation with respect to electrolyte control, the output of individual ions other than sodium, and to a lesser degree chloride, shows no renal defect, and the responses to sharp alterations of ionic load and acid base balance indicate no deviation from normal behavior (Gamble, Charts 29-30). The data here presented might be summarized, then, by the hypothesis that the condition of heart failure with edema consists of a gross enlargement of the extracellular fluid volume, which enlargement somehow fails to elicit the normal hemostatic factors acting on renal tubules. The tubules are behaving, in fact, as normal tubules in the presence of a decreased extracellular fluid volume.

#### SUMMARY AND CONCLUSIONS

Two patients with rheumatic heart disease and congestive failure were studied for a period of three to four weeks by quantitative analysis of all urine electrolytes. The determinations were carried on daily, and the effects of various diuretic salts were evaluated in terms of fluid balance and urine electrolyte pattern.



Estimations of renal filtration rate and blood flow were made by the clearance of mannitol and PAH. In one patient both the filtration rate and the effective blood flow were reduced to approximately 50 per cent of normal. In the second both functions remained well within normal range.

The urine electrolyte pattern of patients in heart failure revealed a constant and specific characteristic, namely an absolute reduction in sodium excreted with a resulting diminution in the ratio of sodium to chloride.

The reduction in the sodium chloride ratio was not associated with any other constant disturbance in electrolyte excretion.

Various mineral salts were effective in promoting diuresis but this effect was of brief duration. Outputs of water and sodium which approximate the control levels were obtained in spite of the continued administration of the diuretic.

Administration of a mercurial diuretic tended to abolish sodium retention and to increase sodium excretion to a much greater degree than it did that of water.

In cardiac edema the glomerular filtration rate may be reduced or it may be maintained within normal limits.

It is proposed that the selective activities described by the data are evidence of normal response by the renal tubules to disturbances involving the regulation of fluid compartments.

We wish to express our sincere appreciation to Professor C. J. Farmer for his generous support and advice in this study.

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# ELECTROLYTE PARTITION IN PATIENTS WITH EDEMA OF VARIOUS ORIGINS

## QUALITATIVE AND QUANTITATIVE DEFINITION OF CATIONS AND ANIONS IN HEPATIC CIRRHOSIS

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WE HAVE previously indicated the probability of sodium retention of a specific nature occurring in congestive failure and to an even greater degree in hepatic cirrhosis<sup>1</sup> With respect to cardiac failure we have described the renal dynamics of salt and water retention in terms of the clearances of mannitol and PAH and the quantitative evaluation of urine electrolytes An abnormal urinary electrolyte pattern was demonstrated which was characterized by sodium retention and a reduction in the ratio of sodium to chloride<sup>2</sup> The purpose of this report is to demonstrate the qualitatively similar but quantitatively even greater retention of sodium and to some extent of chloride in hepatic cirrhosis

### METHODS

Two patients were selected in whom the clinical diagnosis of Laennec's cirrhosis was supported by liver biopsy Both were men, 52 and 54 years of age respectively, jaundice was moderate and ascites was massive. After several days on a general diet the patients were placed on a diet containing a maximum of 15 Gm daily of sodium chloride Mineral and mercurial diuretics were administered as indicated in Tables I and II Paracentesis was done once or twice on each patient, and the electrolyte concentration of the ascitic fluid was determined The cation anion balance was quantitatively itemized from daily twenty four hour collections of urine which were preserved under toluene and refrigerated Analytic methods employed in the determination of ammonia magnesium calcium chloride phosphate sulfate and organic acids are described in the preceding paper<sup>2</sup> Sodium and potassium values were determined by the Beckman flame photometer

A few data were included from a third patient B G, of the same sex and age group in whom complete collections were difficult because of incontinence and illiteracy The urine values are therefore scattered The data are included because of the more regular blood studies available

### RESULTS

The excretion of sodium was found to be exceedingly low with all dietary loads The urinary suppression of sodium, regardless of daily salt ingested was even more refractory to diuretic treatment in these patients than in the patients in congestive failure reported in the foregoing paper

Fluid output was also consistently low and the administration of mercurial diuretics resulted only rarely in a slight diminution in body weight, in contrast with the effect in heart failure

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TABLE I URINE ELECTROLYTES (IN MEQ/24 HOURS), PATIENT D L

DATE (1947)	INTAKE	R <sup>N</sup>	OUTPUT	PH	NA <sup>+</sup>	K <sup>+</sup>	NH <sub>4</sub> <sup>+</sup>	CA <sup>++</sup>	MG <sup>++</sup>	CL <sup>-</sup>	HPO <sub>4</sub> <sup>-</sup>	INORGANIC SO <sub>4</sub> <sup>=</sup>	ORGANIC ACIDS	WT
Nov 7	3320	General diet	750	6.0	1.1	20	33	5.5	3.0	31	6	22	22	187½
8	2380	General diet	805	5.9	1.3	29	55	7.1	2.4	36	25	17	27	191½
9	2500	General diet	1560	6.0	0.9	42	30	4.9	6.4	37	22	13	28	
10		Low salt, Hg†												
11	2364	Low salt	1500	6.1	2.6	61	49	3.2	2.7	51	45	24	28	
12		Low salt, Hg	2040	6.1	4.4	101	23	8.8	9.2	101	24	16	46	193
13	2840	Low salt	1765	6.1	0.7	40	20	2.0	6.9	19	18	16	25	192
14	2620	Low salt, Hg	1285	6.3	1.2	62	22	8.8	10.4	42	14	16	23	191½
15	2500	Low salt	1040	6.2	2.0	38	22	1.6	7.5	12	9	14	21	192
16	2180	Low salt, IIg	1300	6.4	1.5	33	11	4.4	12.5	11	14	9	19	194
17	2780	Low salt†	1165	6.3	1.2	26	16	1.5	6.7	6	23	12	32	194
18	1640	10 Gm potassium acetate, Hg	1720	6.9	28	88	12	1.3	17.3	57	21	11	29	193
19	2380	10 Gm potassium acetate	1450	7.4	1.7	109	6	2.2	5.8	4	28	16	27	194
20	2560	10 Gm potassium acetate, Hg	2665	7.2	4.5	111	9	3.6	10.4	77	6	10	21	195
21*	2080	10 Gm potassium acetate	1580	7.4	3.5	85	7	8.0	5.6	5	14	11	21	196
22	2850	10 Gm potassium acetate, Hg	1590	7.4	1.4	115	5	2.1	5.5	17	37	9	37	176
23	2430	10 Gm potassium acetate	1880	7.4	4	122	19	1.7	10.3	8	30	15	32	179
24	2410	10 Gm potassium acetate, Hg	2215	7.3	1.7	121	6	2.7	8.9	23	25	14	27	180
25	2080	10 Gm potassium acetate	1715	7.4	7	111	6	2.0	7.6	11	27	15	27	179
26	2800	10 Gm potassium acetate, Hg	1500	7.3	1.5	120	4	3.3	10.1	37	23	10	30	177
27	1860	10 Gm potassium acetate	1880	7.3	9	120	4	1.3	6.2	8	25	16	39	179
28	2040	8 Gm KCl, Hg	1720	6.8	21	67	8	4.5	11.5	35	23	12	25	180
29	2260	8 Gm KCl	1700	6.9	6	59	25	2.0	9.8	6	17	13	36	181
30	3240	8 Gm KCl, Hg	1780	6.8	7	85	11	3.5	10.7	36	14	16	37	182
Dec 1		12 Gm NH <sub>4</sub> Cl	1840	6.8	1.7	61	9	4.1	10.5	9	15	13	25	182½
2	3130	12 Gm NH <sub>4</sub> Cl, Hg	3590	6.1	26.2	158	28	8.4	9.9	395	8	13	29	182
3	3120	12 Gm NH <sub>4</sub> Cl	1300	5.9	22	40	59	8.9	11.3	78	2	25	30	180
4	3840	12 Gm NH <sub>4</sub> Cl, Hg	3765	6.1	28.4	117	66	16.0	15.6	437	18	16	34	181
5	2370	12 Gm NH <sub>4</sub> Cl	2340	5.7	8.2	59	106	10.3	8.4	204	31	24	42	177
6	2520	No Medication	1185	6.0	9	27	91	5.3	6.3	66	16	45	24	182
7	2160	No Medication	1425	6.0	3	49	92	7.4	5.5	57	22	54	28	186
8	2860	No Medication	2985	6.2	3	72	125	11.5	18.5	89	61	49	39	179½
9	2380	No Medication	1610	6.2	7.3	116	161	7.5	5.2	277	17	51	32	178

\* 1 mg/kg/day

TABLE II URINE ELECTROLYTES (IN MEQ/24 HOURS) PATIENT J S

DATE (1947)	INTAKE	EX	OUTPUT	PH	NA+	K	NH	CA	MG	CL-	H <sub>2</sub> O =	INORGANIC SO =	ORGANIC ACIDS	WT
No. 9	1100	General diet	600	5.8	17	38	30	0.9	3	16	18	21	1	148½
10	1575	General diet	500	5.7	0.6	33	27	0.9	3.4	6	24	21	21	
11	1400	General diet	900	5.9	7	54	37	1.0	1.0	26	14	10	1	
12	1650	Low salt	350	6.0	--	2	32	0.5	1.1	7	8	13	13	
13	2380	Low salt Hgt†	1465	5.0	81	63	33	99	11.9	182	9	12	15	148½
14	2190	Low salt	763	6.0	30	51	15	33	3	49	2	27	19	145½
15	2250	Low salt	350	5.8	0.3	14	6	0.7	3	3	8		14	152
16	1030	Low salt Hg	1050	5.6	34	66	79	18.9	106	11	11	22	23	151
17	1210	Low salt†	10.0	6.0	4	33	41	2.0	20	23	16	29	25	151
18	1400	8 Gm KCl Hg	1345	5.7	22	61	23	5.4	9.6	70	14	20	28	155½
19	1140	8 Gm KCl	720	6.3	0.7	33	19	0.8	0	3	3	16	20	155½
20	2150	8 Gm KCl Hg	1930	6.4	41	104	27	8.1	5	111	6	16	38	157
21	1040	8 Gm KCl	1340	6.5	1.0	45	23	1.3	7	5	8		20	139
22	1100	8 Gm KCl Hg	1770	6.1	28	143	33	7.3	7.2	1.3	16	20	29	140½
23	1540	8 Gm KCl	2045	6.2	25	39	9	1.4	2.2	17	5	2	9	141½
24	2200	8 Gm KCl Hg	310	6.6	0.8	56	9	1.0	1.0	4	17	16	22	142
25	1580	10 Gm potassium acetate	1555	6.3	0.8	56	9	1.0	1.0	4	17	16	22	144½
26	2000	10 Gm potassium acetate Hg	1290	5.9	4.6	97	15	2.3	2.2	54	3	17	28	144½
27	1640	10 Gm potassium acetate	985	6.0	1.3	49	16	0.9	1.7	8	10		24	133½
28	1900	10 Gm potassium acetate Hg	1690	6.5	0.3	84	20	6.2	6.0	21	15	34	37	135½
29	1600	10 Gm potassium acetate	1015	6.8	1.0	77	21	1.4	3.5	27	6	26	36	137½
30	1450	10 Gm potassium acetate Hg	2750	5.5	34	103	33	7.4	7.8	158	0.6	14	28	159
Dec 1	1710	12 Gm NH <sub>4</sub> Cl	3350	5.7	8	98	35	4.4	3	51	27	21	40	137½
2	2840	12 Gm NH <sub>4</sub> Cl Hg	015	6.1	4	120	47	5.6	6.1	150	6	19	30	
3		12 Gm NH <sub>4</sub> Cl												
4	1710	12 Gm NH <sub>4</sub> Cl Hg	550	5.6	36	18	12	2.7	1.3	74	3	4	6	136
5	2040	No medication	210	5.8	0.3	30	25	1.2	3.7	5	6		8	
7	1215	No medication	500	6.0	2.2	37	20	2.9	2.9	7	7	23	14	134
8	2000	No medication	390	6.1	1.4	5	23	0.9	2.3	6	5	9	9	135½
9		No medication	1000	6.1	0.4	11	7	1.3	4.8	5	8	23	15	

Larcantesis

†Hg ml Mercuhydrin

‡The low salt diet was maintained for the duration of the experimental period

Chloride output was also exceedingly low and tended to be lower than in heart failure. With relation to sodium output, however, the diminution was less extreme, hence the sodium-chloride ratio remained lower than in normal subjects or in congestive failure. The use of mercurial diuretics caused a greater chloride than sodium excretion. This increase in chloride output was in turn frequently associated with an increase in urine potassium.

The excretion of ammonia in response to the administration of ammonium chloride was very different in the two subjects. D. E. showed the typical delayed but protracted increase in urine ammonia (Gamble, Chart 29),<sup>3</sup> and J. S. appeared unable to synthesize ammonia.

Administration of potassium acetate, 100 meq daily, was associated with a low ammonia output, in the cirrhotic subjects as in the cardiac patient to whom the medication was given.

Sulfate excretion tended to be low, a finding which is presumably to be correlated with interference with metabolism of sulphur containing amino acids in the liver.

The excretions of calcium and magnesium were variable in both patients. The determinant of these variations appeared to be chloride rather than phosphate excretion.

#### DISCUSSION

On inspection the data resemble rather closely those derived from the patients in cardiac failure presented in the preceding paper. The initial control periods, without dietary restriction of salt, indicate a retention of sodium, chloride, and water. These positive balances persist notwithstanding the imposition of electrolyte loads by mineral diuretic medication and in spite of the frequent administration of mercurial diuretics. These facts parallel the findings in congestive failure, and establish the fact that in Laennec's cirrhosis sodium retention rather than hypoproteinemia is a major determinant of ascites and edema. Here again, in cirrhosis as in cardiac failure, the pattern is not one solely of filtration of plasma water and electrolytes through the capillary wall, but a more complicated sequence in which the reabsorptive activity of the renal tubules plays a substantial part. As in the cardiac subject, the primary or secondary order of such renal participation becomes a concern of fundamental importance.

While the retention of salt and water lends similarity to the two entities, certain sharp differences appear. First, the urinary sodium concentrations tend to be lower in our three patients with cirrhosis than in congestive failure, indeed, during the control period on a general diet all patients showed a total absence of sodium when the gravimetric procedure was attempted on unconcentrated urine. Although the absolute chloride content was also markedly reduced, it was still high enough to yield a sodium-chloride ratio which was decidedly lower than those found in cardiac patients. The response to mercurial diuretics indicates a more striking divergence. Figs 1 and 2 show only minimal variations in urine volume during the administration on alternate days of Mercurin, and the body weight appears to be unaffected by medical diuretics.

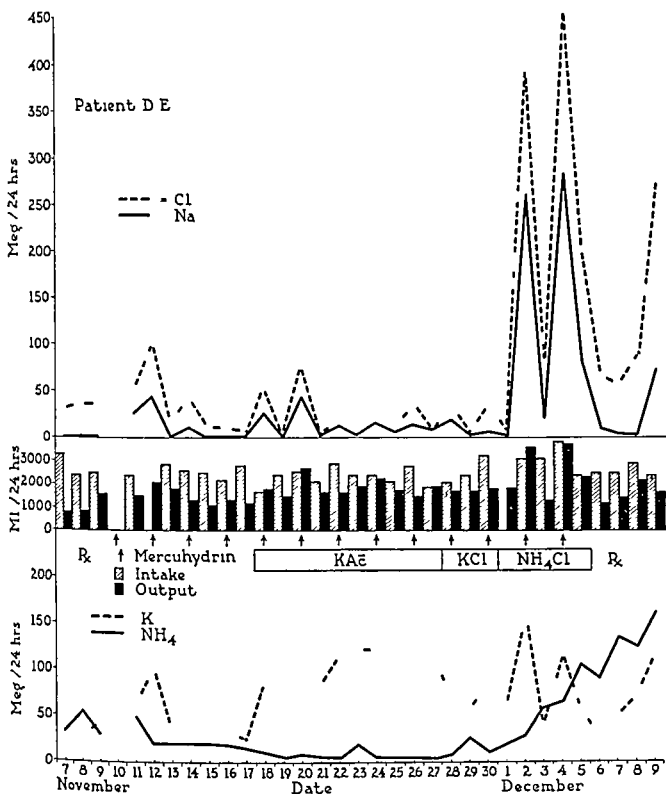


Fig 1—D E electrolyte and water excretion with diuretics

The excretion of sodium is mildly augmented by mercury but, contrary to experience with patients in heart failure the effect on chloride is consistently more marked, and hence the ratio sodium chloride is not restored to normal at any time. These observations pose several questions: firstly, are they the results of incompetent kidneys? second, are they due to abnormalities of electrolyte concentration in one or all of the fluid compartments?

With respect to the functional integrity of the kidneys the clearance rates of mannitol and PAH are given in Table III along with the blood chemistry values. Although substantially below the normal they can hardly be taken to indicate renal decompensation. The fluctuation of urine values of ammonia in

TABLE IV ELECTROLYTE PARTITION IN PLASMA AND URINE, PATIENT B G

DATE	INTAKE	FL	URINE (MEQ/24 HR)					PLASMA (MEQ/LITER)							
			OUTPUT	PH	NA+	K+	NH <sub>4</sub> <sup>+</sup>	CL-	HPO <sub>4</sub> <sup>=</sup>	WT	NA+	K+	CL-	HPO <sub>4</sub> <sup>=</sup>	
May 20		General diet													
24	1700	General diet	160	5.9	0.1	62	18	7	8	146	141	4.9	105		2.6
25	1700	General diet	315	5.8	0.3	23	37	24	14	150					
26	2040	General diet	420	5.8	6	48	46	35	20	151½					
27	1250	General diet	360	6.0	0.6	29	41	24	15	154					
June 1	1500	Low salt	570	5.8	0.3	15	58	15	16	156½					
2*	1300	Low salt	310	5.9	0.6	29	32	6	10	168	131	4.0	105		2.5
8	2500	5% Glucose in water	2400		0.5	7		7	10						
9	5550	5% Glucose in water	1930		0.8	23		8	11	152					
10	3140	5% Glucose in water	2300	6.8	0.4	17		8	9	152	120	4.2	99		2.1
			*Paracentesis												

\*Paracentesis



urine excretion of sodium was almost entirely suppressed. The data given in Tables III and IV show normal concentrations in plasma and ascitic fluid of sodium, potassium and chloride in the case of D. C., with similar values for J. S. with the exception of the plasma sodium of 124 meq. Plasma sodium values for B. G. declined from 141 meq. on a free diet to 120 meq. on a low sodium diet. At those two levels the urine output of sodium was 0.1 meq. and 0.4 meq. respectively. The volume of the extracellular fluid compartment however was grossly enlarged, as evidenced by the massive dependent edema as well as ascites of all patients. Here again a situation exists which would normally elicit a spontaneous diuresis since it is the major function of the renal excretory mechanism to defend not only the concentration but the volume of the extracellular fluid. With respect to salt and water then the kidneys are behaving as if the body were dehydrated. The paradox is essentially the same as was found to exist in the state of heart failure only to a more marked degree.

The findings which we have described support the impression expressed by recent investigators<sup>1, 2</sup> that abnormalities in water distribution in cirrhosis cannot be accounted for solely on the basis of plasma protein deficiency and diminished colloid osmotic pressure.

#### CONCLUSIONS

The urinary electrolyte pattern in hepatic cirrhosis is similar to that in cardiac failure. The renal retention of salt and water long recognized in heart failure, is present to a more marked degree in hepatic cirrhosis.

As in heart failure, the mannitol and PAH clearances may be somewhat reduced, but the metabolic functions of the kidneys are essentially unaffected and other urinary electrolytes exhibit the normal responses to variation in electrolyte intake.

Water and salt diuresis was minimal after administration of mercurial diuretics. One instance is shown however in which a lavish diuresis of electrolytes was initiated by Mercurhydriol.

In addition to the hydrostatic factors operating in cirrhosis the genesis of edema and ascites in this condition is also attributable to a retention of sodium and water. This urinary suppression of sodium is independent of the plasma concentration. Since the competence of the kidneys has been demonstrated by clearance determinations of mannitol and PAH as well as by quantitation of urine electrolytes, the hypothesis is suggested that the stimulus to salt and water retention is specific and that the same stimulus is probably acting in both diseases.

We extend our warm thanks to Dr. A. C. Coreoran for his help in the preparation of this paper.

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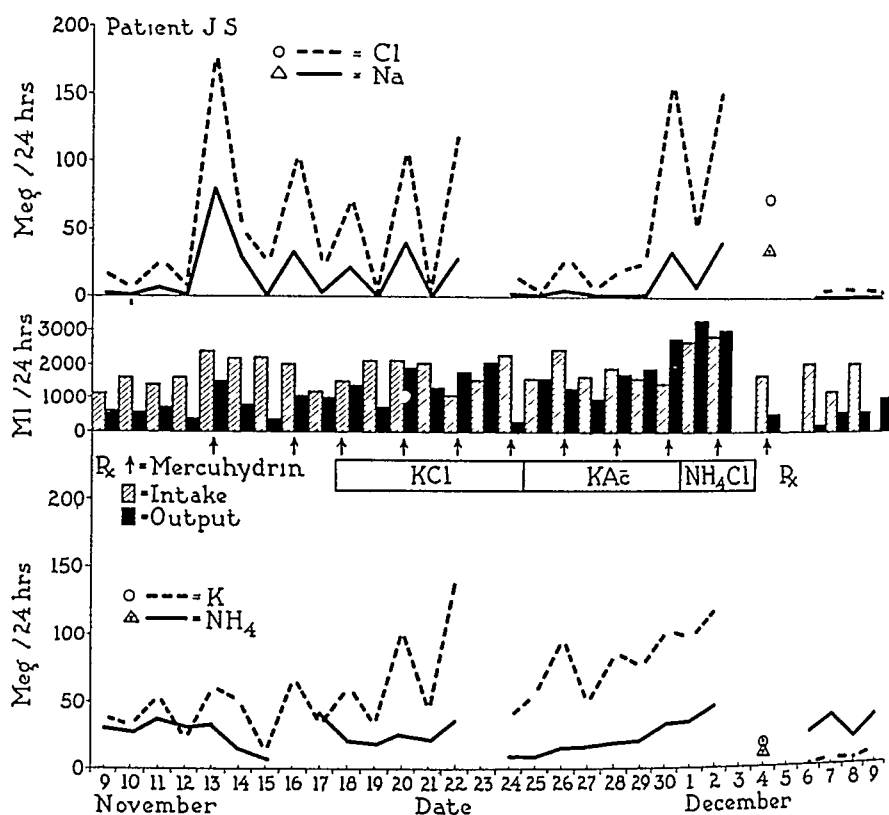


Fig 2—J S electrolyte and water excretion with diuretics

response to ammonium chloride, potassium in response to potassium salts, and sodium in response to ammonium chloride are in themselves renal function tests and offer evidence that the kidneys are not in default. Attention should be called parenthetically to the differences in the two records toward the end of the experimental period. D E tolerated the ammonium chloride, 220 meq daily, for five days, and underwent a dramatic diuresis of electrolytes, whereas J S became comatose after three days on similar amounts of ammonium chloride and the medication was discontinued when the plasma  $\text{CO}_2$  reached 17 meq per liter. Although the urine pH of this patient did not decrease below that of D E under the same conditions, J S appeared to be unable to synthesize ammonia and continued to put out a small volume of urine, poor in electrolytes, in spite of clearance values almost identical with those of D E. The reason for this discrepancy is probably to be found in the poor nutritional condition and extreme anorexia which characterized his hospital course, in contradistinction to D E who ate heartily throughout the period of observation.

The second question which we have proposed concerns the volume and electrolyte composition of the fluid compartments in cirrhosis. In our experience patients with cirrhosis have shown normal or only slightly lowered plasma sodium while on a general diet, reduction in intake has resulted, in several instances, in markedly reduced levels. At all plasma concentrations, however, the

TABLE III RENAL FUNCTION AND PLASMA AND ASCITIC FLUID VALUES IN PATIENTS D E AND J S

	CLEARANCES (ML/MIN )		PLASMA										ASCITIC FLUID (MEQ/LITER)			
			MEQ/LITER					CM/100 ML								
	MANNITOL	PAH	NA	K	CL-	H <sub>2</sub> O	TOTAL PROTEIN	ALBUMIN	GLOBULIN	NA	K	CL-	H <sub>2</sub> O			
D E	74	415	131	38	99	58										
Nov 17																
21																
23							66	0	26	131	57	102	16			
J S							59	55	31							
Nov 12																
20	61	496	124	63	1	106				137	63	107	30			

TABLE IV ELECTROLYTE PARTITION IN PLASMA AND URINE, PATIENT B G

DATE	INTAKE	F.X	URINE (MEQ/24 HR)					PLASMA (MEQ/LITER)						
			OUTPUT	PH	NA <sup>+</sup>	K <sup>+</sup>	NH <sub>4</sub> <sup>+</sup>	CL <sup>-</sup>	HPO <sub>4</sub> <sup>=</sup>	WT	NA <sup>+</sup>	K <sup>+</sup>	CL <sup>-</sup>	HPO <sub>4</sub> <sup>=</sup>
May 20														
24	1700	General diet	160	5.9	0.1	62	18	7	8	146	141	4.9	105	2.6
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2*	1300	Low salt	310	5.9	0.6	29	32	6	10	168	131	4.0	105	2.5
8	2500	5% Glucose in water	2400		0.5	7		7	10					
9	5550	5% Glucose in water	1930		0.8	23		8	11	152				
10	3140	5% Glucose in water	2300	6.8	0.4	17		8	9	152	120	4.2	99	2.1
*Paraentesis														

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## ON THE EFFICACY AND SAFETY OF GLYCINE ADMINISTERED BY VEIN

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THE current rapidly increasing use of amino acid preparations by the intravenous route has suggested a more specific study of certain effects of the simplest of these compounds, glycine, when administered by this method. Although not a significant constituent of the available commercial products since these are principally acid or enzymic hydrolysates of casein which contains little glycine, this amino acid deserves consideration.

Because it is readily synthesized in apparently adequate amounts by the normal human body, glycine falls into the classification of 'nonessential' amino acids—a connotation unfortunately accepted by many to be synonymous with "unimportant" which may explain the lack of attention to this compound in the field of parenteral amino acid preparations. Glycine, however, is a component of many of the body proteins (collagen is rich in glycine), its roles in the syntheses of such nonprotein substances as creatine,<sup>1</sup> glycocholic acid, glutathione,<sup>2</sup> the protoporphyrin portion of hemoglobin<sup>3</sup> and uric acid<sup>4</sup> have received widespread attention and confirmation in isotope studies during the last few years. In each instance the ingestion of glycine tagged with N<sup>15</sup> has been followed so quickly by the appearance of labeled molecules of these products as to indicate the direct participation of this amino acid in their syntheses. Glycine, moreover, has long been known to take a leading part in the detoxication mechanisms applied by the body against a large number of potentially harmful products of metabolism. Representative of this action is the conjugation of benzoic acid, an oxidation product of many toxic aromatic compounds, with glycine to form hippuric acid.

The Quick test of liver function is based on the relationship between the functional state of that organ and the capacity for synthesis of hippuric acid from ingested benzoic acid. It has been shown that supplying extra glycine by mouth during the test will in some instances appreciably increase the hippuric acid output even when it is seriously diminished in liver disease. Thus it is evident that the actual ability of the liver cell to bring about the conjugation may not be impaired, but that the rate of synthesis of glycine is the critical consideration. Depression of this rate occurs in normal pregnancy to the extent of 15 per cent below normal<sup>5</sup> and in toxemias of pregnancy and other conditions the ability to detoxicate is much more seriously impaired.

Here again isotope studies have shed some light upon the mechanism involved. Simultaneous feeding of sodium benzoate and an excess of labeled

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glycine resulted in the utilization of exogenous amino acid for about one third of the hippuric acid synthesis.<sup>8</sup> The body must have supplied the remaining two-thirds required for the detoxication. Very little is known of the source or precursors of this endogenous supply. The observation of Christensen and co-workers that the ingestion of sodium benzoate is followed by a depression of the fasting blood glycine level in human beings<sup>9</sup> has been confirmed in this laboratory. From this it would seem that the body maintains its glycine reserves freely mobile in the blood stream, and depends upon rapid synthesis to meet specific demands for detoxication and other purposes.

Recently, Gubner and associates<sup>10</sup> have employed the specific dynamic action of glycine to bring about some conceivably desirable changes in peripheral blood flow. Their work was based on the correlations that have been established between the metabolic rate and cardiac output and peripheral blood flow.<sup>11, 12</sup> By means of skin temperature recordings, oscillometric readings, plethysmographs, and oxygen consumption determinations they have shown that the increased heat production and oxygen consumption that follow the ingestion of glycine are accompanied by a maximal increase in circulation to the extremities. The skin temperature changes produced are equivalent to those obtained with nerve block and are consistently greater in duration and degree than those caused by alcohol. The presence of peripheral vascular disease in some of their subjects did not alter the response.

This considerable multiplicity of important metabolic functions, viewed in the knowledge that the rate of synthesis of glycine is depressed in a variety of conditions, lends plausibility to the idea that reasonable indications may exist for the supplying of glycine by veins when enteral nutrition is precluded. The studies to follow were undertaken to investigate some effects of glycine administered intravenously.

#### EXPERIMENTAL

For the determination of glycine concentrations in blood and urine, the specific colorimetric micromethod of Alexander, Landwehr, and Sehgal<sup>13</sup> was employed, with the only difference that a Cenco photometer was used instead of the suggested Evelyn or Klett instruments. This method depends upon the distillation conversion of all free glycine into formaldehyde by the action of methylol, the resulting formaldehyde being allowed to react with chromotropic acid to produce a measurable color. Metabolism determinations were made with a standard waterless respirometer.

I. Two female dogs previously fasted for fourteen hours were given infusions of 5 per cent or 10 per cent glycine in physiologic saline solution by simple intravenous drip, the rate of flow being regulated to take thirty minutes. Blood samples were withdrawn immediately before the infusion, immediately afterward, then one and two hours later. Catheterized urine specimens were obtained for the hour preceding administration of glycine and at hourly intervals after the drip began. Two doses were employed: one amounted to 0.5 Gm. of glycine per kilogram of body weight and the other to 1.0 Gm. per kilogram. With the latter dose, both dogs showed signs of distress such as bradycardia, extrasystoles, excessive salivation, and vomiting at the conclusion of the infusion. These signs disappeared within the next half hour, and on a subsequent occasion one of the dogs tolerated this dose without apparent ill effect. Table I is a record of urinary output of glycine during four of the preceding experiments on one of the dogs.



TABLE I. HOURLY OUTPUT OF GLYCINE IN URINE OF A 10 KILOGRAM DOG FOLLOWING INTRAVENOUS INFUSION OF GLYCINE IN SALINE

HOUP	100 ML	5% GLYCINE	100 MI	10% GLYCINE
1	0.2 mg	0.2 mg	0.1 mg	0.2 mg
2	307.3	283.5	1572.5	1252.8
3	18.0	21.0	109.2	146.3
4	3.6	4.2	21.0	38.8
Total	3.89 mg	308.7 mg	1702.7 mg	1436.9 mg

Infusion begun at end of control hour 1 duration thirty minutes

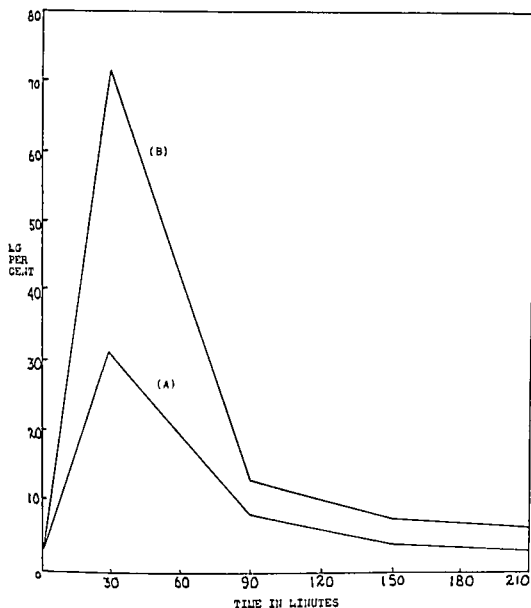


Fig 1 —Blood concentration curves in dog and human being (see text)

II Eight normal adults, fasting for twelve hours were given a total of about twenty intravenous doses of glycine ranging from 12 to 16 Gm on a basis of 0.2 Gm per kilogram of body weight with the method of administration similar to that in the previous experiments. One subject was given a dose of 30 Gm (0.4 Gm per kilogram) and at the end of the half hour injection he noted a feeling of warmth and tingling in the extremities, some increased salivation, slight nausea and lightheadedness, all of which passed within a half hour. No noteworthy subjective manifestations were mentioned by subjects given the smaller dose except the feeling of tingling, which was inconstant.

Blood specimens were taken immediately before and after the infusion and at one, two and three hours thereafter. Duplicate tracings to determine basal metabolism were made before administration of glycine and single tracings were made before each blood withdrawal.

It was soon noted that the curves of blood concentrations obtained on these rather arbitrarily selected doses in dogs and human beings showed striking similarity. Indeed, when average curves for each series were drawn, they could be superimposed for presentation. In Fig 1, curve A represents the average of six closely comparable curves obtained in dogs with the smaller doses (0.5 Gm per kilogram) and of a dozen or more similar curves

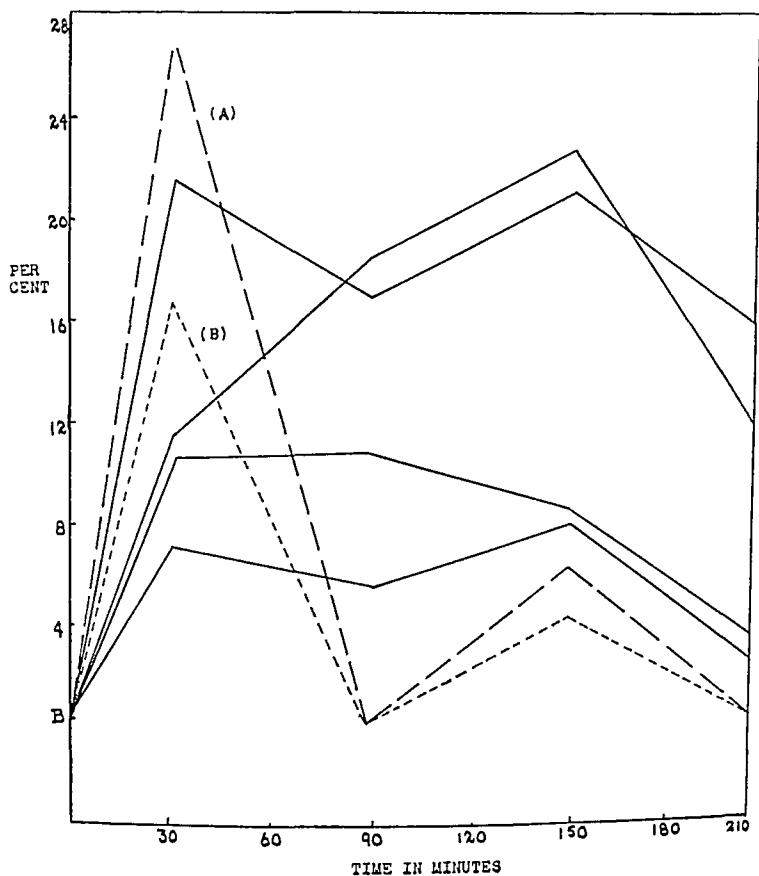


Fig 2—Per cent increase in oxygen consumption (A) following dose of 0.4 Gm per kilogram (B) same subject as (A) after dose of 0.2 Gm per kilogram others after latter dose

from human beings given 0.2 Gm of glycine per kilogram. Curve B was obtained with the doses that produced untoward symptoms in both dog and human being (1.0 and 0.4 Gm per kilogram respectively).

In Fig 2, some curves showing per cent of increase in rate of oxygen consumption above the basal level are presented. A control experiment, using only intravenous saline without glycine, was performed with one student who acted as subject for the glycine experiment on six occasions. This subject showed a perfectly constant rate of metabolism throughout the control test period, but responded to the addition of glycine with increases similar to those presented.

TABLE II HOURLY OUTPUT OF GLYCINE IN URINE OF A 75 KILOGRAM MAN, FOLLOWING INTRA-VEINUS INFUSION OF GLYCINE IN SALINE

HOURLY	150 ML 10% GLYCINE (0.2 GM/KG B.W.)	300 ML 10% GLYCINE (0.4 GM/KG B.W.)
1	20 mg	20 mg
2*	1,720	3,997.6
3	163.5	347.8
4	28.4	35.1
5	4.2	10.0
Total	1,918.1 mg	4,390.5 mg

Infusion begun at end of control hour 1 duration thirty minutes

Urine studies on the subject who received both doses are recorded in Table II, and those on two subjects who received only the small dose in Table III

TABLE III HOURLY OUTPUT OF GLYCINE IN URINE OF 70 AND 80 KILOGRAM MEN FOLLOWING INTRA-VEINUS INFUSION OF GLYCINE

HOURLY	150 ML 10% GLYCINE (0.2 GM/KG B.W.)	150 ML 10% GLYCINE (0.2 GM/KG B.W.)
1	18 mg	25 mg
2*	1,544.4	1,696.2
3	200.7	233.9
4	24.3	31.4
5	7.5	3.5
Total	1,769 mg	1,965.0 mg

Infusion begun at end of control hour 1 duration thirty minutes

III Blood glycine concentration curves were obtained with 0.2 Gm per kilogram dose in two patients with clinical chronic hepatitis (portal cirrhosis). Diagnosis in each case had been confirmed by punch liver biopsy and by abnormal responses to at least three different liver function tests (bromsulfalein, hippuric acid, thymol turbidity and cephalin flocculation). From the data in Table IV it may be seen that the curves were practically identical to each other and to the curve presented as the average of all normal adults. The fasting blood levels are toward the high range of normal but the response to the infusion is strikingly close to the average.

TABLE IV BLOOD GLYCINE CONCENTRATIONS OBTAINED IN TWO PATIENTS WITH PORTAL CIRRHOSIS GIVEN THE 0.2 GM PER KILOGRAM BODY WEIGHT DOSE OF 10 PER CENT GLYCINE IN SALINE COMPARE WITH CURVE A FIG 1

TIME (MIN)	BLOOD LEVELS	
	I	II
0	8 mg	30 mg/100 ml blood
30	32.0	31.0
90	7.6	7.8
150	5.6	5.4
210	5.0	4.5

## COMMENT

The intravenous doses of glycine required to produce signs of distress in dogs (10 Gm per kilogram) correspond closely to those reported by Lewis,<sup>14</sup> Riker and Gold<sup>15</sup> and Loomis and Quick.<sup>16</sup> The dog that showed signs from the dose on one occasion and not on a subsequent trial had a slightly lower minimum blood level on the second occasion (70.0 mg per 100

ml as compared with 740 on the first) It is noteworthy that the blood concentration producing symptoms is similar in the dog and man, although the dose per unit of body weight required to produce them is two and one half times greater in the dog In view of the fact that all subjects tolerated the 12 to 16 Gm (0.2 Gm per kilogram) intravenous doses, even in the short injection time employed, it may be said that this represents a safe dose

Christensen and associates<sup>9</sup> and Gutman and Alexander,<sup>17</sup> the only investigators who have published results of specific determinations of glycine in blood by the method used here, have reported fasting levels slightly lower than those obtained in these experiments Initial levels in their subjects ranged between 18 and 23 mg, averaging 20, while the results presented here are based on fasting levels of 24 to 28, averaging 26 There was a remarkable constancy of this characteristic in individuals tested repeatedly

The apparently "normal" blood level curves obtained in the two subjects with cirrhosis are considered an indication that a "glycine tolerance test" along these lines would have no diagnostic value in this disease, where the deficiency with respect to glycine is in the rate of supply rather than in the metabolism of the amino acid provided exogenously In this connection, however, the work of Weichmann and Dominick on tolerance of intravenous glycine in health and diabetes is worthy of mention They have demonstrated that following the administration of glycine the amino acid nitrogen content of serum returns within normal limits much more rapidly in healthy than in diabetic subjects<sup>18</sup> Insulin caused a faster return Duplication of these experiments, using the specific glycine determination, might produce even more clear cut results

Pitts has shown that of the four amino acids (glycine, alanine, glutamic acid, and arginine) the rate of renal tubular reabsorption is highest for glycine<sup>19</sup> This probably accounts for the strikingly low excretion of unchanged glycine even when the blood concentration is as much as thirty times the fasting level

The abrupt drop in the blood level, without corresponding urinary excretion, during the first hour after the infusion is an indication of the rapidity with which the glycine is metabolized The work involved in this task of deamination and redistribution of the resulting fractions is reflected in the elevated caloric production No previous reports of specific dynamic action of amino acids administered intravenously to human beings could be found in the literature Inasmuch as the necessary total nitrogen metabolism studies were not undertaken, it is not possible to express the results obtained in terms that relate them to the quantity of given substance metabolized, as suggested by Peters and Van Slyke The metabolism effect obtained here with glycine administered by vein corresponds generally in magnitude with that reported by Gubner and associates to follow oral ingestion

Lusk showed conclusively that the increased heat production of specific dynamic action cannot be utilized for the performance of work Nevertheless, the possibility remains that this heightened state of metabolic activity

which can be induced by the administration of a practically nontoxic, readily available amino acid might well be desirable for example, in medical and surgical convalescents. The associated augmentation of peripheral blood flow cannot be ignored. In any condition in which the depression of the body's ability to supply glycine is known or expected (as in preoperative fortification of the liver for biliary tract surgery), the body should profit from parenteral glycine in much the same manner as it does from glucose.

## SUMMARY

Glycine plays an important role in the synthesis of body protein creatine, glycochole acid, glutathione, uric acid, and heme. It is apparently essential in detoxication and exhibits remarkable versatility in general metabolism. The possibility of depression of glycine synthesis in abnormal states should be recognized.

Blood and urine studies following the intravenous administration of glycine are presented to demonstrate the rapidity of its metabolism and its lack of toxicity even in high blood concentrations. Specific dynamic effect is obtained when it is given by this route.

As an adjunct to the usual parenteral protein therapy glycine may be administered intravenously with safety and with beneficial effect when oral feeding is not possible.

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# DETERMINATION OF ANTIBODY CONTENT OF LYMPHOID CELL EXTRACTS

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BETHESDA MD

RECENT investigations<sup>1</sup> of the role of lymphocytes in antibody formation have focused attention on the technical difficulties of serologic titration with small volumes of cells. The present work represents an investigation of the requirements of serologic technique using the small cell volume available from the lymph or lymph nodes of experimental animals.

The lymph was obtained from the efferent vessel of the popliteal node of a rabbit. The cell content of the lymph was determined using a white blood cell pipette and a standard counting chamber as used for peripheral blood examination. The total cell volume was estimated by the formula of Harris and co-workers<sup>1</sup> in which cell volume in milliliters equals the cell count in thousands times 0.002 times the number of milliliters of lymph. The total cell volume available in our experiments varied from 0.001 to 0.01 milliliter. It was felt that the smallest volume that could be pipetted with accuracy in this work was 0.05 milliliter. Employing the principle of Hemolytic System Adjustments<sup>2</sup> the following procedure for diluting lymph extracts was followed using 0.2 ml pipettes graduated in 0.001 ml and test tubes 10 by 75 millimeters.

TUBE	SALINE	FACTOR
1 0.05 ml substance	---	1
2 0.05 ml substance	0.05 mix remove 0.05 ml to control tube	2
3 0.05 ml substance	0.1 mix discard 0.1 ml	3
4 0.05 ml substance	0.15 mix transfer 0.05 ml to tubes 5, 6, 7	4
5 0.05 from tube 4	0.05 mix discard 0.05 ml	8
6 0.05 from tube 4	0.10 mix discard 0.10 ml	12
7 0.05 from tube 4	0.15 mix transfer 0.05 ml to tubes 8, 9, 10	16
8 0.05 from tube 4	0.05 mix discard 0.05 ml	32
9 0.05 from tube 4	0.10 mix discard 0.10 ml	48
10 0.05 from tube 4	0.15 mix transfer 0.05 ml to tubes 11, 12, 13	64

To each tube 0.05 ml of *Salmonella typhimurium* antigen (density equal to McFarland tube number 5) was added. Vials were shaken and incubated in a water bath at 37° C for two hours, were left in the cold room overnight and read the following morning.

A comparison of this method of dilution and the usual macromethod of employing 0.2 ml of material in serial dilution in 0.2 ml of saline is given on the following page.

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Dil	MACRO								MICRO							
	10	20	40	80	160	320	640		10	20	30	40	80	120	160	320
Serum																
1	4	3	2	+	+				4	4	4	3	±	±		
2	4	4	3	+	+				4	4	4	3	+			
3	4	4	4	4	4	+++	±		4	4	4	4	4	4	+	
4	4	4	4	+	±				4	4	4	3	2	+	±	
5	4	4	4	4	±				4	4	4	3	2	+	±	

Lymphoid cell extracts were prepared by freezing and thawing, with and without preliminary washing of the cells. After the lymph had been drawn, and the count taken, the rest of the lymph was measured into a small test tube and centrifuged at 2,000 revolutions per minute for five minutes to sediment the cells. The lymph was withdrawn with a capillary pipette and saved for testing, the cells remained in the same tube. To the estimated volume of cells, a volume of saline was added which would give sufficient material for testing with a minimal dilution factor for the cells. Lysis of the lymph cells to extract antibodies was effected by freezing with dry ice and thawing at 37° C. This process was repeated three times. A clear supernatant was obtained by centrifugation and used in the serologic test outlined.

In addition, experiments were carried out in which the lymph was divided into two portions. One portion was treated as previously outlined. In the procedure with the other, instead of extracting the cells in the saline, they were gently resuspended and centrifuged, and the saline was removed and saved for testing. The cells were resuspended in a like volume of saline and subjected to the lysing process.

In the example of results given below, the rabbit had been injected in the left foot pad with 1 cc of *S. typhimurium* suspension equal in density to McFarland tube number five.

Antibody titer of unwashed cells of lymph from right popliteal node	1 102
Antibody titer of unwashed cells of lymph from left popliteal node	1 70
Antibody titer of washed cells of lymph from right popliteal node	Negative
Antibody titer of washed cells of lymph from left popliteal node	1 13
Antibody titer of saline wash of right cells	1 10
Antibody titer of saline wash of left cells	1 13
Antibody titer of right lymph	1 160
Antibody titer of left lymph	1 160
Antibody titer of serum	1 480

It appears that the apparent high titer of extracts of cells of lymph may be entirely or in large part due to small amounts of lymph adherent to unwashed cells, or to the sides of the tube. Therefore these effects were investigated further.

Four tenths milliliter of a serum with a titer of 1 512 was placed in a small test tube and the serum was carefully removed with a capillary pipette. A volume of 0.05 ml saline was delivered into the same test tube and removed with a capillary pipette, whereupon a second volume 0.05 ml volume of saline was added. In serologic measurements the first saline wash was positive in a



dilution 1:256, and second wash was positive in a dilution of 1:12. Had the titer of the saline been calculated in reference to a hypothetical volume of cells (0.0025 ml) which had been frozen and thawed to extract immune bodies the titer of the first wash would have been 1:2856 the second wash 1:324. Hence the presence of traces of fluids containing immune bodies greatly distorts titers which are computed with reference to extracts of extremely small volumes of cells.

Again multiple washes were used to eliminate the effects of traces of body fluids in an investigation of the extracts of cells of the popliteal node. The detailed procedure was as follows: The popliteal node was weighed and placed in a small beaker with a volume of saline nine to nineteen times its weight. The node was cut sufficiently to permit the release of the cells yet leave the node tissue in one minced section rendering filtration unnecessary. After a cell count had been taken the cell saline suspension was measured into a small test tube and centrifuged (1000  $\times$  gravity) for five minutes. The supernatant was carefully withdrawn (referred to below as wash 1) and saved for testing. Droplets of fluid observed on the sides of the test tube were removed with a clean cotton swab. Employing the same test tube the volume of cells (computed as count in thousands  $\times$  volume in milliliters  $\times$  0.0002) was resuspended in fresh saline an amount which was a multiple of the volume of cells and gave sufficient material for testing. After this second suspension had been centrifuged the supernatant was withdrawn and saved as wash 2.

The cells were then suspended in an amount of distilled water equivalent to half of wash 2. After the cells had been lysed through the combined effects of distilled water and alternate freezing and thawing a volume of 17 per cent sodium chloride solution equal to the amount of distilled water was added. The mixture was shaken, allowed to stand then centrifuged. The microtechnique was used to estimate the amount of antibody present in the cell extract as well as the washes. A typical result is presented.

Wash 1 titer 1:80 (proportion computed for weight of node)

Wash 2 titer 1:30 (proportion computed for volume of cells)

Cell extract titer 1:16 (proportion computed for volume of cells)

#### SUMMARY

The present work represents an investigation of the requirements of serologic technique using the small cell volume available from lymph or lymph nodes of experimental animals.

The experience of this laboratory indicates that especial care must be exercised in the interpretation of titers because small errors of fluid containing antibodies may greatly distort results if these are expressed in terms of cell volume.

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# THE EFFECT OF NITROGEN MUSTARD AND X IRRADIATION ON BLOOD COAGULATION

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IN PRODUCTION

ALLEN, Jacobson, and associates<sup>1, 4</sup> reported that exposure to ionizing radiations produced a prolonged whole-blood coagulation time (Lee-White) in dogs and in human beings as a result of the appearance in the blood of an anti-coagulant biologically similar to heparin. More recently Smith, Jacobson, and co-workers observed a prolonged whole-blood coagulation time in five patients given therapeutic doses of nitrogen mustard (methyl-bis-( $\beta$ -chloroethyl) amine hydrochloride). This clotting defect was identical to that reported by Allen and co-workers in the dog and human being following exposure to ionizing radiation in that the clotting time could be returned to normal both in vivo and in vitro by specific antiheparin substances such as toluidine blue and protamine sulfate. The prothrombin time and the calcium and fibrinogen blood levels were within normal limits in these subjects but each had a severe leucopenia and thrombocytopenia.

Reports by Jacobson and associates,<sup>6</sup> Spurr and co-workers,<sup>7</sup> and Block and co-workers<sup>8</sup> have pointed out the toxic effects of nitrogen mustard on the blood and blood-forming tissue of patients who were given this drug therapeutically. Although these authors reported pancytopenia, a prolonged bleeding time, ecchymosis and cutaneous and mucous membrane petechiae, no alteration of significance in the whole-blood coagulation time was described.

The dosage and schedule of administration of nitrogen mustard in the five patients referred to were as follows. Two patients were given 0.1 mg per kilogram of body weight intravenously on four consecutive days. The injections were administered twenty-four hours apart and the total dose in one was 25.4 mg and in the other 26.8 milligrams. One patient received four injections of 0.1 mg per kilogram of body weight at intervals of twelve hours, the total dose was 22.4 milligrams. One patient was given four injections of 0.1 mg per kilogram at intervals of seven hours, the total dose was 20.0 milligrams. One patient was given two injections of 0.3 mg per kilogram, each six hours apart, the total dose was 3.8 milligrams. The fact that a severe hemorrhagic state, terminating fatally, developed in all of these five patients led us to suspect an impurity or some intramolecular transformation in the drug or unusual sensitivity of the patient to the drug. However, other pa-

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tients treated with the same lot number of the drug and with similar total dosage did not manifest this clotting defect nor has a new supply of this drug produced such a toxic reaction.

Because of the rather widespread clinical use of nitrogen mustard in the treatment especially of neoplastic diseases of the hemopoietic system and the serious implication of hemorrhage, studies in experimental animals were undertaken to elucidate further this effect.

#### MATERIALS AND METHODS

Swift's snuffle free rabbits of uniform age and weight (2.5 to 3 kilograms) were used in this study. Previous studies with nitrogen mustard in our laboratories<sup>9</sup> indicated that a single intravenous dose of 30 mg. per kilogram of body weight produced a destructive effect on the hemopoietic system of this strain of rabbits approximately equivalent to 800 r total body X irradiation. The effect of both of these agents on the whole blood coagulation was studied simultaneously for comparative purposes.

*Technique of Nitrogen Mustard Administration*—A dose of 3 mg. of nitrogen mustard per kilogram of body weight was injected slowly into the marginal ear vein of the rabbit immediately after the chemical had been dissolved in physiologic saline. A concentration of 1 mg. of the drug per 1 cc. of saline was used since this concentration produced no sloughing of the ear tissue and did not obliterate the ear vein.

*Technique of X Irradiation*—The X rays administered in these experiments were generated on a 200 kv. machine operating at 15 milliamperes. A 0.5 mm. copper filter and a 1 mm. aluminum filter were used. The half value layer in copper of the filtered beam was 0.98 millimeter. The exposure was measured with a Victoreen condenser meter equipped with a 100 r. chamber. Measurements were made in air within a treatment box at the position occupied by the center of the animal's body. Victoreen chambers of 250 r. full scale readings were used as monitors.

#### Hematologic Studies—

*Whole blood Clotting Time*—The clotting time of the whole blood was determined at room temperature by a modification of the Lee White method.<sup>10</sup> Whole blood (0.5 cc. amounts) was measured into five or six small test tubes (10 by 75 mm.) and 1 cc. amounts were pipetted into two or three large test tubes (13 by 100 mm.). The larger tubes were not examined until clotting had been established in the small tubes. The time which elapsed between obtaining the blood (first appearance in the syringe) by cardiocentesis and the clotting of two consecutive tubes which had not previously been manipulated was considered to be the whole blood clotting time, and the blood was considered to be clotted when it remained firm upon inversion of the tube.

*Platelet Determination*—Platelet counts were made by drawing blood to the 0.5 mark in a whole blood diluting pipette and diluting to the 11 mark with a 1.8 per cent solution of freshly prepared sodium citrate. The pipette was gently shaken for one minute after which the entire content of the pipette was allowed to flow into a small Lusteroid conical shaped tube. The tube was set aside at room temperature until sedimentation of the red cells had occurred. The platelet counts were then made on the plasma whenever time permitted (within twenty four hours).

TABLE I AMOUNTS OF PROTAMINE SULFATE AND BLOOD USED IN THE  
HEPARIN TOLERANCE TEST

TUBE	1	2	3	4	5	6	7	8	9	10
γ Protamine sulfate	5.0	10.0	15.0	17.5	20.0	22.5	25.0	27.5	30.0	35.0
Heparinized blood* (c.c.)	0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Amount of protamine sulfate† (c.c.)	0.01	0.02	0.03	0.035	0.04	0.045	0.05	0.055	0.06	0.07

0.05 c.c. liquid heparin in 3.5 c.c. blood  
11 mg. protamine sulfate in 2 c.c. normal saline

*Heparin Tolerance Test*—Modifications of the heparin tolerance technique developed by Allen and co workers<sup>4</sup> were necessary to avoid the repeated withdrawal of large amounts of blood from rabbits. Protamine sulfate which was weighed on an analytical balance was dissolved in physiologic saline in a concentration of 1 mg of protamine to 2 cc of saline. This solution, which was prepared every other day, was titrated into a series of ten small test tubes (10 by 75 mm) as shown in Table I. In performing the test, 3.5 cc of blood obtained by cardiocentesis were immediately added to a tube containing exactly 0.02 cc of liquid heparin and gently inverted twenty times. Immediately thereafter, 0.25 cc of this heparinized blood was added to tubes containing the graduated amounts of protamine sulfate described. Each tube then was shaken gently and set aside at room temperature. At the end of one, three, and twenty four hours, readings were made to determine the minimum amount of protamine sulfate necessary to cause the blood to clot. Normal rabbit blood requires 17.5 to 22.5  $\gamma$  protamine to bind the heparin in the amounts used. With one exception, the one hour readings are reported in this communication.

*Prothrombin Studies*—Prothrombin determinations were made according to the Link Shapiro modification of Quick's method.<sup>11</sup> Commercial thromboplastin (Maltine) and both diluted and undiluted blood plasma were used in these determinations.

*Calcium and Fibrinogen*—Calcium determinations were made on the blood serum by precipitating the calcium as an oxalate according to the method of Clark and Collip.<sup>12</sup> The presence of fibrinogen was noted by heating the plasma to 58° C in a water bath. No quantitative fibrinogen determinations were made.

#### GENERAL PROCEDURE

Rabbits were divided into three groups for the nitrogen mustard experiments. Group I received nitrogen mustard (3 or 4 mg per kilogram of body weight) intravenously. Group II received 3 or 4 cc of physiologic saline per kilogram of body weight intravenously. Group III consisted of normal untreated animals that received neither nitrogen mustard nor saline. Since saline was used as a solvent for the nitrogen mustard, Group II animals that received saline served as a control for Group I, while Group III which received neither nitrogen mustard nor saline served as a control for both Groups I and II.

Approximately 10 cc of blood were withdrawn from the rabbit by cardiocentesis using a dry 18 gauge needle and a dry 10 cc syringe. Three and one half cubic centimeters of this blood were immediately transferred to a graduated centrifuge tube containing 0.02 cc of liquid heparin for the heparin tolerance test, 0.9 cc of blood was transferred to a tube containing 0.1 cc of sodium oxalate for the prothrombin determination, the remaining blood was pipetted in 0.5 and 1 cc amounts into a series of test tubes for the whole blood clotting time as previously described.

Only one attempt was made to obtain blood from the heart of the rabbit. When that was unsuccessful no further attempts were made to obtain blood until the animal had rested (at least two hours).

After a prolonged clotting time and an abnormal heparin tolerance test had been established, one group of animals was given toluidine blue (64 per cent dye content) intravenously, while another group was treated with protamine sulfate. Both toluidine blue and protamine sulfate were given in concentrations of 1 mg per 1 cc saline. The doses of protamine or toluidine blue given per injection were 3 and 4, and 2 and 2.5 mg, respectively, per kilo

gram of body weight. One group of animals that received 3 mg of nitrogen mustard per kilogram of body weight remained untreated for comparative purposes.

Animals exposed to 800 r total body X radiation were treated similarly, one group receiving either toluidine blue or protamine after a prolonged clotting time had been established, while another group remained untreated.

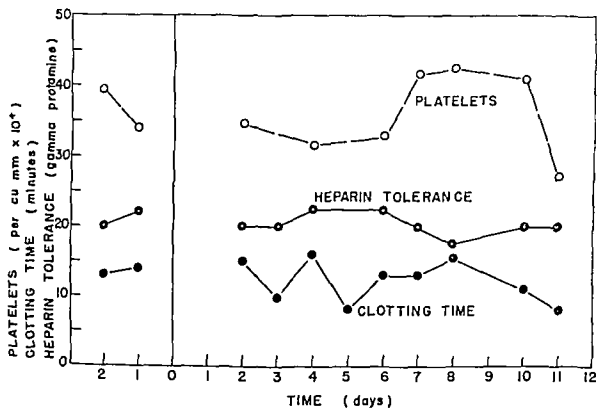


Fig 1A—Average clotting time (1 cc blood) platelet values, and heparin tolerance test (one hour results) in normal rabbits (blood obtained by cardiocentesis)

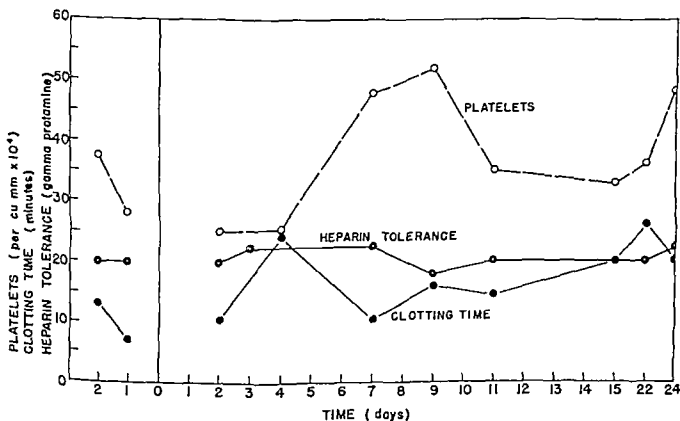


Fig 1B—Clotting time (1 cc. blood) platelet values and heparin tolerance test (one hour results) in one normal control rabbit (O.S.) (blood obtained by cardiocentesis)

## RESULTS

The mean average of the whole-blood clotting time in normal untreated rabbits varied from 8 to 16 minutes in tubes containing 1 c.c. of whole blood. The platelets of the normal rabbits varied considerably but were usually between 250,000 to 500,000 per cubic millimeter. The heparin tolerance test gave fairly constant results in normal rabbits, 17.5 to 22.5  $\gamma$  of protamine sulfate were necessary to produce a clot in heparinized blood prepared as outlined. Thus clotting occurred normally in tube 4, 5, or 6 (see Table I). The mean average results of the whole-blood clotting time, platelets per cubic millimeter, and the heparin tolerance results of seven untreated normal animals are shown in Figs 1A and 1B. Fig 1A illustrates the fluctuations in these determinations which a single animal may exhibit during a twenty-four day period of observation. Normal animals on which such determinations are made at frequent intervals over a period of thirty days or more usually exhibit less variation than this individual animal illustrates.

*Effect of Nitrogen Mustard—*

**Clotting Time**—A prolonged clotting time of the whole blood occurred after the intravenous injections of 3 or 4 mg. of nitrogen mustard per kilogram of body weight. Fig 2 shows the effect on the clotting time of sixteen rabbits given intravenous doses of 3 mg. per kilogram. An increase in the length of the clotting time was present twenty-four hours after nitrogen mustard administration, reached a peak in *circa* four to seven days, remained high about ten days, and returned to a normal value in the surviving animals by two weeks. The effect of nitrogen mustard on the clotting time of an individual animal is shown in Fig 3. The pattern of individual response is similar to the mean values shown in Fig 2 except that the clotting time was not infrequently prolonged to 50 and 60 minutes in individual animals.

**Platelets**—The data from sixteen animals studied consecutively after nitrogen mustard administration are shown in Fig 2. The mean maximum decrease in the platelets per cubic millimeter had occurred by the fourth day after nitrogen mustard administration, and remained below normal values for *circa* seven days. The lowest mean platelet level (150,000 per cubic millimeter) did not parallel the high peak in the clotting time on the seventh day, but the return of the clotting time to normal limits paralleled the return of the platelets to normal values. Even more striking is the fact that the clotting time in practically all animals had increased prior to a significant reduction in platelet values. These facts are clearly illustrated in individual animals as is shown in Fig 3.

**Heparin Tolerance**—In general, the effect of nitrogen mustard on the heparin tolerance paralleled the effect on the clotting time. As the clotting time of the animal rose, a greater concentration of protamine sulfate was necessary to produce a clot in the heparin tolerance tubes in one hour. This is illustrated in Figs 3 and 6.

**Fibrinogen and Calcium**—Without exception fibrinogen was present in all plasma samples. The blood calcium determinations of the nitrogen mustard

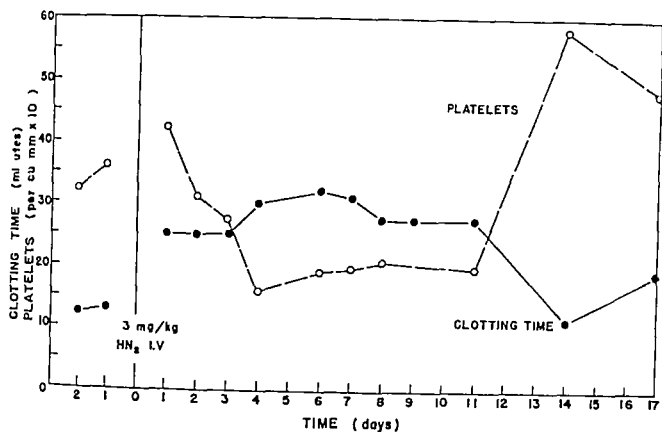


Fig 2—Clotting time (1 c.c. blood) and platelet values in sixteen rabbits following the intra venous administration of 3 mg per kilogram of nitrogen mustard

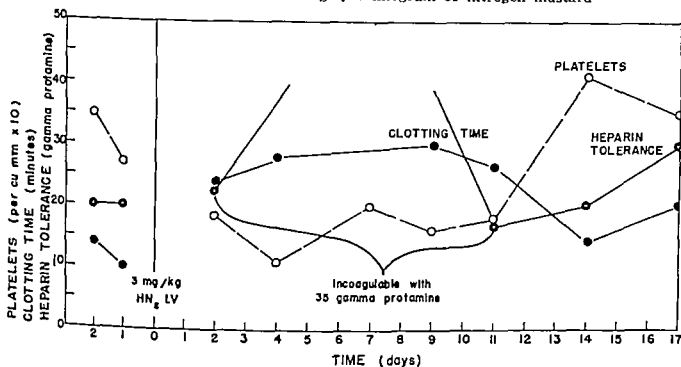


Fig 3—Clotting time (1 c.c. blood) platelet values and heparin tolerance test (one hour readings) in one rabbit (0.33) following the administration of 3 mg per kilogram nitrogen mustard

treated animals with delayed clotting and an abnormal heparin tolerance test were within the normal range (13 to 14 mg per cent)

**Prothrombin Time**—As stated, blood for a prothrombin time determination was obtained simultaneously with blood for clotting time and heparin tolerance determinations in all control and mustard injected animals. In no instance was a prolonged prothrombin time observed in animals which had a prolonged clotting time or abnormal heparin tolerance test as a result of the nitrogen mustard intoxication.

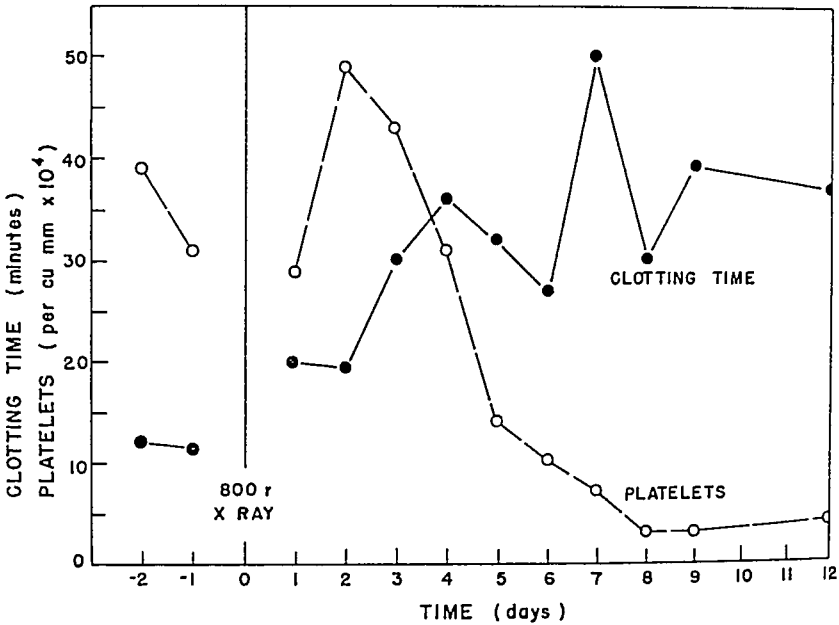


Fig 4—Average clotting time (1 c.c. blood) and platelet values in sixteen rabbits after 800 r total-body X irradiation

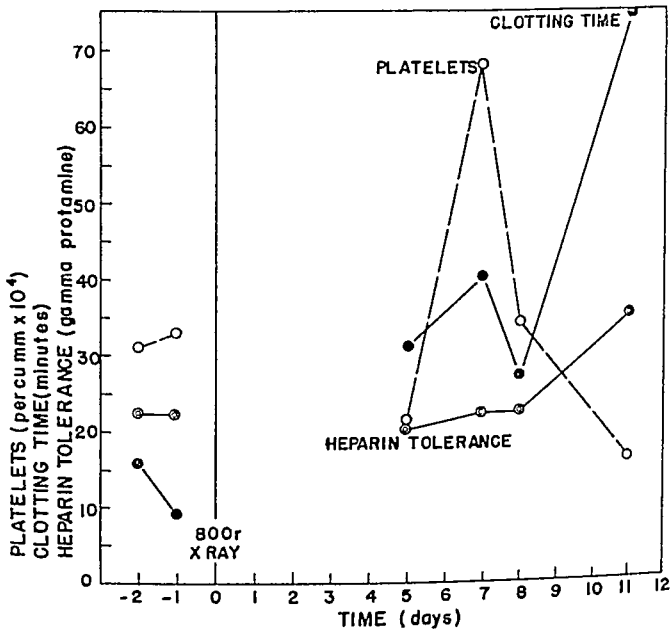


Fig 5—Clotting time (1 c.c. blood) platelet values and heparin tolerance test (one hour results) in one rabbit (0762) after 800 r total-body X irradiation

*Effect of X-Rays*—Exposure to 800 r total-body X radiation produced results similar to those described following nitrogen mustard administration except that the prolonged clotting time persisted for a longer period of time and the platelets, which were reduced by the fifth day, remained reduced for a period of seven days or longer. Fig 4 is representative of this group





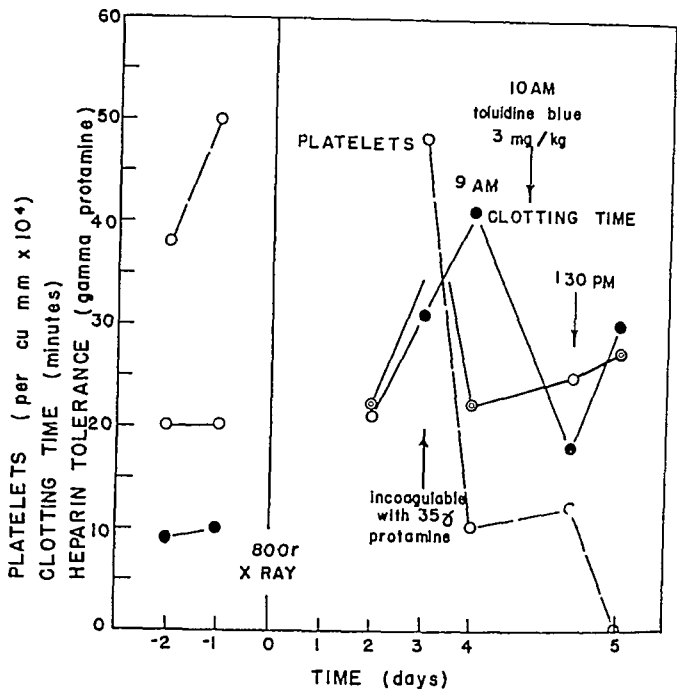


Fig 7A—The effect of toluidine blue on the prolonged clotting time (1 c.c. blood) platelet count and heparin tolerance (one-hour readings) in a rabbit (0780) following 800 r total-body X irradiation

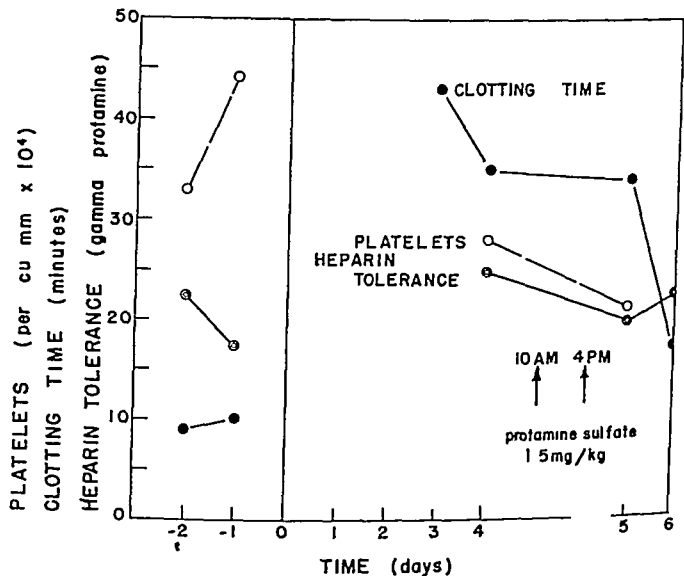


Fig 7B—Effect of repeated doses of protamine sulfate on the prolonged clotting time platelet values and heparin tolerance in a rabbit (0764) following 800 r total body X irradiation

sary to produce a clot in the heparin tolerance test was reduced from 25 to 10  $\gamma$  during the same period. The intravenous injection of 3 mg of toluidine blue per kilogram of body weight gave similar results in the same rabbit six days after the first injection of protamine when the clotting time was again prolonged. After the toluidine blue injection, the clotting time dropped from 65 minutes to 42 minutes in two hours, and then to 35 minutes in twenty-four hours. Another injection of 4 mg of toluidine blue reduced the clotting time to 22 minutes in two hours, after which the animal made an uneventful recovery.

No significant change was noted in the number of platelets per cubic millimeter following the injections of toluidine blue or protamine sulfate.

Without exception in the presence of a nitrogen mustard induced prolongation in the clotting time, the intravenous injection of toluidine blue in 3 or 4 mg per kilogram doses or of protamine sulfate in doses ranging from 15 to 25 mg per kilogram significantly reduced the clotting time. In some instances the clotting time was reduced to the normal range by two hours or significant reduction in the clotting time was produced by two hours but was not reduced to the normal range. In others, the clotting time was significantly reduced by two hours, reached the normal range twenty-four hours after injection of toluidine blue but by forty-eight hours was again prolonged.

In rabbits exposed to 800 r total body X irradiation the effects of toluidine blue and protamine sulfate on the prolonged clotting time are shown in Figs 7A and 7B. Although the clotting time was reduced in rabbit number 0780 (Fig 7A) from 41 minutes to 18 minutes in two hours after the intravenous administration of 3 mg of toluidine blue per kilogram of body weight the effects on the heparin tolerance test were not remarkable. The following day the clotting time was again prolonged (30 minutes) and an increased amount of protamine was necessary to bind the heparin in the heparin tolerance test. Before another injection of toluidine blue could be administered the animal died. Occasionally as in this animal an abnormal heparin tolerance fails to revert to normal two hours after the intravenous administration of an antiheparin substance even though the clotting time was returned to normal or significantly reduced. By twenty-four hours after toluidine blue or protamine injection however the heparin tolerance was normal if the clotting time had returned to normal.

Animal number 0764 received protamine sulfate in 15 mg per kilogram amounts intravenously at 10 AM and 4 PM when the clotting time was 43 minutes and when 35  $\gamma$  of protamine did not clot the blood in the heparin tolerance test. The following day when the clotting time was 34 minutes another injection of protamine (1 mg per kilogram) was given intravenously. Although the clotting time was reduced to 17 minutes in twenty-four hours after the last injection of the protamine sulfate the animal did not survive. It is likely that these animals died as a result of the cardiocentesis or from the general  $\gamma$  irradiation effects rather than from the protamine or toluidine blue injections.

## DISCUSSION

A prolonged clotting time and an abnormal "heparin tolerance" were almost invariably produced in rabbits given a single intravenous dose of 3 mg per kilogram or more of nitrogen mustard (methyl-*bis*-( $\beta$ -chloroethyl) amine hydrochloride). A similar effect was produced in rabbits after exposure to 800 r whole-body X radiation. The prolonged clotting time appeared by twenty-four hours in rabbits given nitrogen mustard, whereas significant prolongation in clotting time was not consistently observed until the second or third day after irradiation. Spontaneous recovery from this effect occurred earlier in the nitrogen mustard treated animals than in irradiated animals. The effects of a single injection (30 mg per kilogram) of nitrogen mustard on the cellular elements and hemoglobin of the peripheral blood and the histologic effect on the hemopoietic tissues are essentially comparable with those produced by 800 r whole-body X radiation. Recovery of the bone marrow and lymphatic tissue and return of the cellular constituents of the peripheral blood to normal values after doses of these two agents is more rapid in nitrogen mustard treated animals than in the irradiated animals.<sup>9</sup> Since, however, these two agents are not strictly comparable in terms of selectivity and mechanism of action, one cannot expect the appearance of or recovery from largely identical biologic effects to be the same.

The mechanism of the prolonged clotting time and altered heparin tolerance, which appears after nitrogen mustard intoxication, is probably identical with that described by Allen and Jacobson<sup>2</sup> in the dog after exposure to lethal amounts of X radiation, namely an increase in a circulating heparin or heparin-like substance. This is substantiated by the facts that the prothrombin time, fibrinogen, and calcium levels were normal in the animals with a prolonged clotting time, no fibrinolysis was demonstrated, and antiheparin substances, such as toluidine blue and protamine, were capable of reversing to normal values or significantly reducing the prolonged clotting time for varying lengths of time. The recent report by Smith, Jacobson, and co-workers<sup>5</sup> has shown that therapeutic doses of nitrogen mustard produced this same effect in the human being. It is of interest in this connection that the dose required to produce this clotting defect in the rabbit with regularity is about seven times the total dose usually used for therapeutic purposes in the human being.

In the original publication by Allen and Jacobson<sup>2</sup> it was pointed out that not infrequently a prolonged clotting time was observed in X-irradiated dogs before thrombocytopenia developed. In the rabbit, after intoxication with either nitrogen mustard or x-ray, the clotting time is almost always significantly increased prior to significant reduction in platelet values. On the other hand, the maximum increase in clotting time roughly parallels or occurs concomitantly with the maximum platelet decrease. Spontaneous recovery of both to normal levels occurs at practically the same time. Reversal of the clotting time and heparin tolerance to the normal range by the intravenous administration of toluidine blue or protamine sulfate did not significantly

alter the platelet level. These facts do not necessarily indicate, however, that platelets have no role in the production of this "syndrome".

The results of these experiments are interesting from the standpoint that a clotting defect is produced by nitrogen mustard which is similar to that produced by ionizing radiations. It seems likely that other chemical substances with the capacity to produce severe damage to the blood-forming tissue may likewise produce a prolonged clotting time in which heparin or a heparin-like substance is involved. It is an important fact that spontaneous recovery from this hemorrhagic syndrome produced by either nitrogen mustard or irradiation is possible. It is also significant that antiheparin substances are therapeutically effective in the treatment of this syndrome.

#### SUMMARY AND CONCLUSIONS

A prolonged clotting time is produced in rabbits by the intravenous administration of 3 or 4 mg of nitrogen mustard per kilogram of body weight. The same syndrome is produced in human beings after therapeutic doses of this drug. The amount of protamine necessary to produce clotting in the heparin tolerance test is increased in those rabbits that develop a prolonged clotting time.

The prolonged clotting time occurs in rabbits after a 3 mg per kilogram dose within twenty-four hours after administration and persists for approximately twelve days. Spontaneous recovery occurs in surviving animals by the twelfth to fourteenth day. A maximum reduction in blood platelets occurs by the fourth day in rabbits receiving 3 mg per kilogram nitrogen mustard, with recovery by the twelfth to fourteenth day. Thus the clotting time is prolonged prior to significant platelet reduction. However, the period of platelet reduction (fourth through twelfth day after nitrogen mustard administration) roughly parallels the period in which maximum increase in the clotting time exists. Spontaneous recovery of the clotting defect and the return of platelet values to normal levels in the peripheral blood occur at a comparable time (*circa* fourteen days).

Animals which developed a prolonged clotting time had normal calcium and prothrombin values.\*

The prolonged clotting time and decreased "heparin tolerance" are reversible with antiheparin substances. The anticoagulant present in the blood is probably heparin or a heparin-like substance.

The clotting defect produced by nitrogen mustard resembles that produced by whole body X irradiation (800 r) in rabbits and is also reversible with antiheparin substances.

It is suggested that the potentially serious clotting defect may be produced by other agents producing toxic effects directly or indirectly on the blood-forming tissue. This should be borne in mind since a method of treatment is available (antiheparin substances) which may make it possible to pass the critical period of potential hemorrhage until spontaneous recovery ensues.

\* Since submission of the manuscript, quantitative fibrinogen determinations have been done on a few animals in which a prolonged clotting time was present after nitrogen mustard administration. In these animals the fibrinogen values were normal.

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## THE USE OF PLASMODIUM VIVAX PRESERVED BY FREEZING IN INDUCING MALARIA

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FEVER from induced malaria continues to be widely employed in the management of neurosyphilis, particularly in parenchymatous forms such as dementia paralytica and primary optic atrophy. A serious obstacle to the use of malaria has been the difficulty of maintaining a source of parasites. Even in metropolitan areas, cases of malaria naturally acquired or deliberately induced are frequently not available. This difficulty has increased since the introduction into syphilotherapy of penicillin. Milder forms of neurosyphilis are now treated effectively with this antibiotic alone, consequently treatment with induced fever is less frequently necessary and fewer cases of malaria are to be found. It may be troublesome and time consuming to obtain malarial blood from distant medical centers and unless shipment is rapid and delivery prompt the blood may be noninfectious by the time inoculation is accomplished. Since malarial parasites remain viable at room temperature for three to five days at most, a simple effective method for their preservation for longer periods has practical value.

By a method of quick freezing of malarial blood and storage at low temperatures, we have had available a supply of *Plasmodium vivax* parasites which has been used successfully during the past year to induce malaria in approximately forty patients in hospitals in the St. Louis area.

A preliminary report<sup>1</sup> briefly outlined the method used and cited successful inoculations in six patients, three of which were described in detail. It is the purpose of the present paper to record the experience with a larger series of cases.

It is well known that many infectious agents bacterial and viral can be maintained in the frozen state. As summarized in our previous communication several types of pathogenic protozoa, including those of avian and monkey malaria have been similarly preserved.<sup>1</sup> The only previously recorded attempt at preserving human plasmodia was that of Coggeshall<sup>2</sup> who was unsuccessful using a method which involved rapid freezing at  $-72^{\circ}$  to  $-80^{\circ}$  C followed by slow thawing. The procedure employed in the present study is fundamentally similar except that thawing has been effected rapidly rather than slowly.

### ORIGIN OF PARENT STRAINS

*H K Strain*—This strain was isolated April 8 1947 from a 29 year old veteran during an acute episode of relapsing malaria. H K had been exposed in the Southwest Pacific area, New Guinea and the Philippines, from June 1944 to December 1945. Throughout this time while on routine quinine suppression no clinical attacks of malaria occurred.

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Suppressive therapy was stopped in December, 1945, when he returned to the United States, a few weeks later he suffered his first acute attack of malaria which was followed by repeated relapses, about ten in all, between February, 1946, and April, 1948. Blood first was drawn for preservation on April 8, 1947, after three paroxysms, the last one having occurred four to six hours before venipuncture. Blood films showed numerous amoeboid and younger forms of *P. vivax* with a parasite density of about 10,000 per mm<sup>3</sup>. During the course of the next year, partly through oversight and the more frequent use of another preserved strain of *P. vivax*, the H K strain was lost, but was recovered in April, 1948, when H K again suffered a relapse. Blood drawn a few hours after a shaking chill when the patient's temperature was 39.2° C showed many young ring forms, about 20,000 per mm<sup>3</sup>.

*N Y Strain*—This strain was isolated June 13, 1947, from a 29 year old war veteran on the first day of a relapse. While serving in the Philippines during 1944-1945, N Y suffered his initial attack of malaria. Since discharge from the Army he had been having relapses every two to three months. At the time of blood withdrawal numerous young ring forms of *P. vivax* were present on smear, a parasite count indicated about 5,000 per mm<sup>3</sup>.

#### METHOD OF PRESERVATION\*

Blood from malarial donors was prevented from clotting by the use of sodium citrate or by defibrination with glass beads. Citrate was employed in a 4 per cent solution in a ratio of 5 parts of blood to 1 part of sodium citrate, giving a final concentration of less than 1 per cent of the salt. A part of the citrate was drawn into the collecting syringe in order to eliminate possible coagulation in the syringe, then the required amount of blood was drawn and the contents of the syringe were added to a flask of 125 ml capacity containing the remainder of the citrate. Defibrination was accomplished by adding the blood to a flask containing a layer of glass beads and shaking or rotating the contents gently until the fibrin collected in a mass about the beads.

After citration or defibrination the blood was transferred in 2 to 4 ml amounts to thin walled glass ampules, about 15 by 80 mm in size, and the ampules were sealed by flaming. The ampules were then immersed in a previously prepared freezing solution consisting of ethyl alcohol and dry ice at a temperature of -70° to -80° C. Ampules were immersed individually while being rotated rapidly in order to disperse the contained citrated blood into a thin film on the inner surface of the ampule. Freezing was thus accomplished in the course of a few seconds. After remaining in the freezing mixture approximately ten minutes, the ampules were transferred to a dry ice box for storage at -70° C. A commercial home freezer with a specially constructed insulated inner cabinet containing a central space for storage of ampules and compartments at the ends for dry ice has proved satisfactory for this purpose. The entire process, from venipuncture to storage, was completed as rapidly as possible, usually requiring no longer than thirty to forty five minutes. Aseptic precautions were observed throughout.

Ampules were removed as needed, rapidly thawed at the bedside by immersion in a water bath at 40° C, and the fluid immediately was injected intravenously into the recipient. The usual inoculum consisted of the contents of two or three ampules, a volume of 4 to 10 ml, although as little as 2 ml has been used successfully.

#### TRANSFER OF STRAINS

Following the development of clinical malaria, patients were bled after varying numbers of paroxysms and during various phases of the life cycle of the parasite, and the freezing and storage process was repeated. Thus, the parent strains were maintained by serial passage from one patient to another. Inoculations were performed uniformly with material which had been frozen and resort to direct transfer was not necessary.

\*Although the method described includes minor modifications adopted during the course of the study the procedure has remained essentially unchanged.



# DESCRIPTION OF SUBJECTS

Since the beginning of the experiment a little more than a year ago a total of fifty four subjects has been inoculated with preserved *P vivax* blood one subject being inoculated twice, for a total of fifty five inoculations. All subjects were patients with neurosyphilis in whom malaria was being induced for therapeutic purposes. All were adults forty eight were men and six were women, all were white except one Negro and one Mexican of mixed blood. The racial selection was premeditated since it is well known that Negroes are frequently immune to vivax malaria. The patients were hospitalized in ten different hospitals in the St. Louis area.

# RESULTS

Results of inoculation have been classed as successes failures incomplete data and lost to observation, according to the following criteria. An inoculation was considered a success when it was followed by oral temperatures of  $37.8^{\circ}\text{C}$  or more on at least one occasion forty eight hours or more following inoculation in addition to the demonstration of malarial parasites in thick or thin blood preparations. In successful cases the incubation period was calculated as the time in days from inoculation to the first febrile elevation.

Cases were officially classed as failures when parasites were not found in the blood during the period from about six days to four or five weeks after inoculation. However, two patients who had irregular low grade fever on the tenth to fourteenth postinoculation days without parasites being demonstrated subsequently developed typical malarial paroxysms with demonstrable parasites one forty five days and the other seventy eight days after inoculation. Hence it seems likely that some of the patients who were classed as failures after four to six weeks and who were then lost from observation or given antimalarial therapy might have developed malaria. Cases classed as incomplete include those patients whose postinoculation period is currently less than four weeks without clinical or laboratory evidence of malaria and two patients who were discharged from hospitals less than three weeks after inoculation who developed chills and fever at home, recorded temperatures exceeding  $39^{\circ}\text{C}$  and who promptly became afebrile following antimalarial therapy blood smears not being available for examination during the posthospital period. In the two cases lost to observation the patients left the hospital less than a week following inoculation and subsequent observations are lacking.

Of the fifty five inoculations there are at present forty six cases with data adequate to classify as success or failures. Table I shows that forty or 87 per cent of the forty six, developed malaria, and six or 13 per cent were classed as failures. These six failed to develop malaria clinically and parasites were never demonstrated by smear over observation periods ranging from thirty four to ninety days.

TABLE I. RESULTS OF INOCULATION OF *P VIVAX* STRAINS PRESERVED BY FREEZING

RESULT	STRAIN N. Y.	STRAIN H. L.	TOTAL
Successes	34	6	40
Failures	4	2	6
Total	38	8	46
Incomplete	1	5	6
Lost	2	0	2
Total	41	13	54

Sufficient information relative to possible strain differences has not been accumulated thus far. Among the cases for which we have adequate data, of thirty-eight inoculations performed with N Y strain, thirty, or 90 per cent, have been successful, six, or 75 per cent, of eight inoculated with H K strain developed malaria.

In considering causes of failure other than technical error, three factors deserve mention—previous malarial infection, race, and the inadvertent suppression of the malaria by drugs possessing antimalarial activity. Whether any of the six known failures had suffered previously from naturally acquired malaria and might, therefore, have been immune is not definitely known. Two of these patients, so demented that historical information on this point could not be obtained, had lived in malarious regions. One was a Greek who had lived in his native country for an eighteen-year period, the other was a Mexican who was also probably part Negro. A third patient, classified as a failure after forty-five days of observation following an initial inoculation with the H K strain, subsequently was reinoculated successfully with the N Y strain, the incubation period (sixteen days) being within the usual range. In this patient the original inoculation was probably faulty since it seems less likely that strain differences would have influenced the result. Failure may be explained in the case of the one Negro subject on the basis of race resistance.

None of the forty who developed malaria are known to have had the naturally acquired disease, but one had been previously inoculated (unsuccessfully) with fresh blood containing quarian parasites.

In reference to antimalarial drugs, it is well known, although perhaps not widely recognized, that both the sulfonamides and the heavy metal antisypilitic agents,\* including the arsenicals and bismuth, may abort or suppress infection with malarial parasites. The same effect may be produced by even minute doses of quinine comparable to those contained in some of the commonly used "tonics." To our knowledge, none of the failures were related to the administration of these drugs, although several of these patients were not under our own observation. It may be of interest to refer to one patient who developed severe convulsive seizures a few days after inoculation and a day or two after penicillin therapy was instituted. To control the convulsions the patient was given regular doses of phenobarbital, and Dilantin sodium, 90 mg three times a day. It was felt that his failure to develop malaria might have been due to some suppressive action of the barbiturates or Dilantin since it is known that many types of complex organic compounds may act as antimalarials. To test the supposition that Dilantin might act in this manner, one patient in whom it was desired to terminate malaria was given Dilantin sodium, 90 mg three times a day for a period of four days. He suffered a regular malarial paroxysm on the first day of Dilantin treatment but was afebrile subsequently for five days, during which malaria parasites were not found in blood smears. At this time the patient was given a course of chloroquine to make sure the malarial infection was terminated. In another patient, similar doses of Dilantin for three days failed to have any apparent effect upon the malaria, and because of his critical condition the patient was treated with chloroquine to end the attack.

\*But not penicillin

In attempting to analyze the results of inoculation there are a number of variable factors, some of which may be controlled and measured with reasonable accuracy, while others are beyond control and cannot be measured with present methods. The measurable, controllable factors include parasite strains, the number of parasites in the inoculum before freezing, the stage of development of the parasites, the method of preventing clotting, the speed of freezing and thawing, the storage temperatures, and the duration of preservation. The uncontrollable, incalculable factors include the host resistance whether due to previous malaria or other factors including race.

*Parasite Strains*—The differences in results of inoculations with the two strains used in this study including the proportion of successes to failures and the incubation period in the successes, are too small to be of significance.

*The Size of Inoculum*—It might logically be assumed that few parasites might fail to produce malaria, or produce it only after a prolonged incubation period, and that a large number of parasites would produce malaria more consistently and with a relatively short incubation period. In most instances a rough parasite count was made on donor's blood by relating the number of parasites found in stained smears to the number of leucocytes seen and to the total leucocyte count. But, although we can thus estimate the number of parasites in a given quantity of blood before freezing, it has not been possible to determine how many survive the freezing, thawing, and storing.

There is no evidence that the total number of parasites in the inoculum before freezing accounted for success or failure in producing malaria in the host. In the six cases classed as failures the number of parasites was greater on the average than in the cases classed as successes and the blood had been preserved for shorter periods of time. Furthermore when the successes are grouped according to number of parasites in the inoculum as shown in Table II, there is no evidence that the incubation period was altered significantly by increasing the number of parasites. The length of time the parasites were stored was essentially comparable in all groups except the fourth in which there was a much longer average storage time and a slightly shorter incubation period.

TABLE II THE SIZE OF INOCULUM, THE LENGTH OF STORAGE OF PARASITES AND THE INCUBATION PERIOD IN CASES OF SUCCESSFUL INOCULATION

SIZE OF INOCULUM NUMBER OF PARA- SITES IN MILLIONS (PER MM <sup>3</sup> )	TIME FROZEN IN DAYS		INCUBATION PERIOD IN DAYS		NUMBER OF CASES
	MEAN	EXTREMES	MEAN	EXTREMES	
9.19	31.7	10-102	12.8	9-14	7
21.40	31.1	6-85	12.6	10-22	14
41.69	54.4	6-166	11.3	7-16	12
100.137	26.5	3-59	15.0	12-20	4

*The Stage of Development of Parasites*—It is possible that the resistance of parasites to freezing and thawing and prolonged storage at low temperatures might vary with their age, size, and complexity of structure that is, with the stage of development. As a working hypothesis it was assumed that the merozoites, or very young trophozoites, might be the most resistant, and the mature

TABLE III THE STAGE OF DEVELOPMENT OF PARASITES RELATED TO THE INCUBATION PERIOD

STAGE OF DEVELOPMENT OF PARASITES IN INOCULUM	THE INCUBATION PERIOD IN DAYS				TIME FROZEN IN DAYS		SIZE OF INOCU- LUM, MILLIONS OF PARASITES PER MM. <sup>3</sup>	EXTREMES OF CASES
	MEAN	MEDIAN	MOD.	EXTREMES	MEAN	EXTREMES		
Young trophozoites	11.9 ± 3.74	11.5	10.7	6.22	44.0	6.131	9.100	20
Mature trophozoites	13.9 ± 2.77	14.0	14.2	10.20	18.0	3.47	9.137	8
Schizonts	12.3 ± 2.37	12.0	11.4	10.16	42.9	9.166	19.46	9

trophozoites and schizonts, the least resistant. If that hypothesis were correct then we would expect more successes and shorter incubation periods when using the younger forms. We have tried to draw blood from donors at times in relation to the developmental cycle to provide young, half grown or mature forms of the parasites. However, since merozoites are beginning to be liberated probably hours before the mass disruption of schizonts both young and old forms of parasites may be present in considerable numbers near the end of the cycle and differences in results might be expected to be less from samples taken early and late than from those taken early and mid way in the cycle.

It is not possible to decide whether age of parasites had any effect on the proportion of successes to failures because the numbers are too few. Successes and failures both followed inoculation of young, middle aged or old parasites. When successes alone are considered there is suggestive evidence that the incubation period was shorter when the inoculum contained a majority of young ring forms than when the majority were mature trophozoites. This is shown in Table III, which indicates that when young ring forms predominated the incubation periods were grouped around eleven days, when mature trophozoites predominated, around fourteen days, and when schizonts predominated with some young trophozoites the incubation periods were grouped around twelve days. In other respects the groups are fairly comparable except that the average time frozen was much shorter in the mature trophozoite group.

It is always hazardous to draw conclusions from small differences which may exist between small groups, but apparently there is a significant prolongation of incubation period in the mature over the young trophozoite groups. The difference between the means is  $2 \pm 0.737$ , the difference being nearly three times the standard error. The significance of the difference is further borne out by a comparison of results following inoculation of one group of seven patients with blood from donor M<sub>1</sub> with another group of four patients inoculated with blood from H<sub>0</sub>. The size of inoculum and duration of preservation were essentially equal and the blood had been citrated in both cases. Blood from M<sub>1</sub> showed parasites approximately forty hours old, schizonts while that from H<sub>0</sub> taken just at the time of the paroxysm showed a majority of parasites to be very small ring forms with a number of mature schizonts. In the M<sub>1</sub> group the incubation period averaged 12.7 days with a range of ten to sixteen days while in the H<sub>0</sub> group the incubation period averaged 7.7 days with a range of six to ten days.

*Anticoagulation*—Citrated blood was used in thirty two cases with successes resulting in 27 or 84 per cent and defibrinated in 14 cases with 13 or 93 per cent resulting in malaria. This is not a significant difference. In the cases in which malaria developed there was no apparent significant difference in incubation periods in the two groups which were comparable in other ways.

The speed of freezing and thawing was as nearly identical as possible in all cases and the conditions of storage were the same in all.

*The Length of Period of Storage*—There is no evidence that the length of time the parasites are kept frozen affects the final results of inoculation. In comparing successful with unsuccessful inoculations it is found that failures

followed freezing periods ranging from 11 to 137 days, and successes followed freezing periods ranging from 3 to as long as 166 days. Other factors in the two groups were essentially comparable. In considering only successful inoculations, Table IV indicates that the incubation period does not change as the duration of freezing increases.

TABLE IV THE LENGTH OF STORAGE OF PARASITES IN RELATION TO THE INCUBATION PERIOD AND SIZE OF INOCULUM

STORAGE PERIOD IN DAYS	INCUBATION PERIOD IN DAYS		SIZE OF INOCULUM, PARASITE COUNT IN MILLIONS PER MM <sup>3</sup>	NUMBER OF CASES
	MEAN	EXTREMES	EXTREMES	
3 10	12 2	9 16	9 64	6
11 20	13 2	10 20	9 137	11
22 28	12 0	10 16	13 225	7
37 59	12 1	6 17	13 100	7
70 166	12 1	8 22	17 69	7
3 166	12 4	6 22	9 225	38

*Incubation Period*—In thirty-eight patients who developed malaria, the incubation period, when it could be accurately determined, varied between six and twenty-two days, the mean being 12.4 days. This would seem to be somewhat prolonged over that following the direct transfer method. Moore<sup>3</sup> stated that with the latter using an inoculum of 1 to 10 ml. the average incubation period is three to eight days. We have had only one opportunity to draw a comparison between the two methods. Using direct transfer the incubation period in one recipient was forty hours, with the same blood preserved and inoculated into a different patient, 10 days. It seems likely that the destruction of many plasmodia as a result of the freezing and thawing process, rather than a host factor, was responsible for the prolongation of the incubation period.

The success or failure of attempts to produce malaria in equally susceptible subjects with blood preserved by freezing and the incubation period must depend upon the number of viable parasites surviving. Although available data are insufficient, we have the impression that one important factor in determining the number of parasites surviving is the stage of development, and that merozoites or young trophozoites are more resistant than older forms.

Evidence also has been obtained that infectiousness is not affected adversely by repeated freezing. One strain has been passed serially through six patients, the preservation process being repeated each time, without a significant change in incubation period. Repeated freezing and thawing of the same specimen of blood have not been attempted.

*Reactions to Inoculum*—The preservation process results in almost complete hemolysis of the red blood cells, smears after thawing show only an occasional intact cell. The staining properties of the malarial parasites also appear to be affected for, after thawing, the latter are poorly stained by either Wright's or Giemsa's method. Despite these rather marked alterations resulting from the preservation process, no untoward effects directly attributable to the inoculum have been observed following intravenous injection of the relatively small amounts (4 to 10 ml.) employed.

*Clinical Course of Malaria*—The clinical course of the disease resulting from inoculations with preserved parasites has in no way differed from that seen following the direct inoculation of whole blood. Striking individual differences in the severity of the disease have been observed: some patients exhibiting a low parasitemia and tolerating the infection well. Others have had fulminating infections, high parasite indices with red blood cells containing as many as three to five parasites, terminating in peripheral circulatory collapse which necessitated interruption of treatment. This individual variation to infection is believed to be related to host rather than to parasite factors. There have been no fatalities in this series of cases.

#### CONCLUSIONS

1 Preservation of viable parasites of vivax malaria for many months by a simple method of quick freezing, storage at low temperature ( $-70^{\circ}$ ), and quick thawing has provided a constantly available supply of parasites in the St. Louis area during the past year.

2 Among forty-six subjects inoculated with preserved blood forty or 87 per cent, subsequently developed malaria.

3 The incubation period in those cases of malaria in which an accurate determination was possible averaged 12.4 days, ranging from six to twenty-two days. Malaria was first proved in two additional subjects, forty-five and seventy-eight days after inoculation, although both had slight temperature elevations at ten to fourteen days at which time parasites were not found in blood films.

4 The incubation period probably is directly related to the number of parasites surviving preservation which in turn appears to be inversely related to the age of parasites; younger forms of parasites are apparently more resistant to freezing and thawing.

5 The clinical course of malaria does not appear to differ in any way from that following infection with fresh parasitized blood.

The authors wish to thank Dr. Louis Kohler, Superintendent of the Missouri State Hospital and especially Dr. Leopold Hofstetter of the same institution and Dr. Alfred K. Baur of Jefferson Barracks Hospital for their assistance in providing subjects for inoculation.

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## LABORATORY METHODS

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### SPUTUM CELL STUDY FOR PULMONARY CARCINOMA AS A ROUTINE LABORATORY TEST

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**A**LTHOUGH the examination of sputum for neoplastic cells as an aid in the early diagnosis of carcinoma of the lung has become well established,<sup>4, 11</sup> it is still not being carried out in most laboratories as a routine procedure. The reasons for this hesitancy are understandable. Fear of the harm that might be wrought by a false positive diagnosis, the feeling that specialized cytologic knowledge is required, and the long time frequently necessary for the adequate study of a single case have acted as deterrents. Thus, while there are at present a few laboratories in which this technique for early diagnosis of cancer is being applied very successfully, it is not yet available to most physicians.

To stimulate more widespread use of this valuable diagnostic aid, perhaps a more practical approach to this problem is needed. It has been our experience that the great majority of positive sputum diagnoses are based on cells which have easily recognizable neoplastic characteristics. It was suspected that by confining positive diagnoses only to the relatively obvious cases, the sputum examination might prove to be more adaptable to routine use in a clinical laboratory without fear of false positive diagnoses, and at the same time retain a useful degree of accuracy.

To test this suspicion, a group of forty consecutive cases of proved carcinoma of the lung, in which sputum examinations were done, were analyzed. It was found that in twenty-nine cases (72 per cent) the sputum contained obviously malignant cells. While this percentage of positive results is lower than that reported by some authors,<sup>4, 6</sup> it is still high enough to be of distinct clinical value. Thus, in seven of the twenty-nine cases in which we demonstrated neoplastic cells in the sputum, bronchoscopy had been entirely negative, and histologic proof of the suspected presence of a lung tumor could not be secured by any other means short of exploratory thoracotomy. It would appear that by restricting positive diagnoses only to those patients whose sputa contain easily identifiable malignant cells, and by reporting all others as negative, the sputum examination should become practical for more general adoption. This paper outlines the technique of preparing sputum slides, describes and illustrates the cellular elements encountered, and sets forth the characteristics of the obviously malignant cells.

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## TECHNIQUE

The technique has been described in other publications<sup>10</sup> but certain points bear emphasis. The proper collection of the specimen is one of the most important steps in the sputum examination. Due care exerted in this part of the procedure will be rewarded by noticeably better results and will save the examiner a great deal of time spent in unfruitful search of specimens containing almost no bronchial secretion. The patient must be instructed to cough up material from the tracheobronchial tree and must not be allowed to present the examiner with saliva or nasopharyngeal secretions as a specimen. Material from the patient's bedside sputum cup is usually not satisfactory, due to the dilution of whatever bronchial secretions there are with a relatively large amount of saliva. We have found it advisable to have a well-instructed technician or nurse obtain the specimen directly from the patient.

*Storage*—It is best to smear and fix the specimen soon after collection if possible. However, when necessary a specimen may be allowed to remain in the refrigerator for four to six hours without appreciable deterioration.

*Preparation of Slides*—The specimen is poured into a Petri dish placed on a black background. An effort is made to find small fragments of tissue and blood tinged material. Samples of the various components of the sputum are selected and placed on a glass slide. A fairly thin and uniform film is obtained by smearing the material with a second glass slide. Ordinarily five slides are prepared from each specimen.

*Fixation*—Fixation of the film is accomplished by immersion for at least thirty minutes in a mixture of equal parts of ether and 95 per cent alcohol. It is important for the preservation of cellular details that no drying of the film be allowed to occur before it is fixed. The slides should be placed in the fixative while still wet.

*Staining*—The stain employed is hematoxylin and eosin. This stain presents two important advantages over other stains that have been advocated for use in this work. First it allows the staining of sputum slides to be carried out simultaneously and by the same technique as other routine slides. Second it relieves the pathologist of the necessity of familiarizing himself with a new stain. From the ether alcohol mixture the sputum slides are transferred to 95 per cent alcohol for one minute and from then on are handled as are ordinary tissue sections. In our laboratory this consists of the following:

- 1 Water for one minute
- 2 Harris hematoxylin for one minute
- 3 Water for one minute
- 4 Dip in acid alcohol
- 5 Water for one half minute
- 6 Ammonia water till blue
- 7 Water for one half minute
- 8 One half per cent aqueous eosin for two minutes
- 9 Dip in water
- 10 Dip in increasing concentrations of alcohol to absolute alcohol
- 11 Beechwood creosote for five minutes
- 12 Xylol five minutes

The slides are then coverslipped with the use of Clarite. The preparation of the slides, fixation and staining, can easily be handled by a technician. The examination of the slide consists of scanning with the low power objective using a mechanical stage so that all parts of the preparation are seen. Suspicious elements are studied with the high dry objective.

## NORMAL CELLS IN SPUTUM

All sputum specimens, regardless of the underlying pulmonary disease contain normal cells which can be classified according to their place of origin.

nuclei as to render adequate study difficult and to make it impossible to appreciate hyperchromasia, because all the nuclei, benign or malignant, will appear dark blue. One of the important criteria of malignancy is thus lost.

Another possible source of error may be the presence of degenerating normal cells. The changes brought about by this process include hyperchromasia and swelling of the nucleus, vacuolization of the cytoplasm, and changes in size and shape of the cell. Degenerating normal cells may thus assume what appear to be characteristics of malignancy. But careful observation will disclose loss of distinct cellular and nuclear outlines and loss of nuclear detail. Frequently, in the close vicinity one finds similar but better preserved benign cells, and others which are obviously and more completely degenerated. Occasionally macrophages which have no visible ingested material in their cytoplasm and whose nuclei are rather prominent are encountered. Such cells may simulate undifferentiated neoplastic cells, or, if they contain vacuoles, cells arising from adenocarcinoma. However, phagocytes occur only as individual cells and are not hyperchromatic, and similar cells with ingested material can usually be found in the immediate vicinity.

In chronic infectious diseases the normal bronchial epithelium may undergo squamous metaplasia (Plate I, 7). Fragments of the metaplastic epithelium are frequently exfoliated into the sputum and may resemble bits of well differentiated squamous carcinoma. However, these cells are arranged in an orderly fashion, have a definite polarity, and usually do not contain visible nuclei. As a result of degeneration, they may show both hyperchromasia and variation in size and shape of the nuclei. However, the cause of these characteristics becomes evident when one notes the loss of nuclear structure, distinct nuclear outline, and vacuolization of the cytoplasm.

Occasionally one is tempted to make a positive diagnosis on the basis of one or two single cells which have all the characteristics of malignancy. It is wise in such instances to withhold the final diagnosis until further specimens can be examined. In almost every case one will eventually find either numerous other similar cells or small fragments of tumor tissue. It has been shown that the examination of three separate sputum specimens will yield about 96 per cent of the positive diagnoses.<sup>2</sup> However, if the clinical history and roentgenographic studies strongly indicate malignancy, and especially when bronchoscopic biopsy is impossible, as in upper lobe lesions,<sup>1</sup> it is often desirable and worth while to repeat the examination several more times.

The diagnosis given to the clinician should be concise and explicit. We feel that the grouping of cells as to the probability of malignancy is confusing and even dangerous. Unless the pathologist is absolutely certain that carcinoma cells are present in the sputum, he should render a negative report. Care should be taken to advise the clinician as to the significance of positive and negative reports. The presence of cancer cells in the sputum is not a priori evidence of primary bronchogenic carcinoma. The cells may arise from carcinomas of the mouth, pharynx, nasopharynx, larynx, esophagus, trachea, or from pulmonary metastases, and in such instances cannot be differentiated from cells of primary bronchogenic neoplasms. Above all, one must be aware of the fact that failure

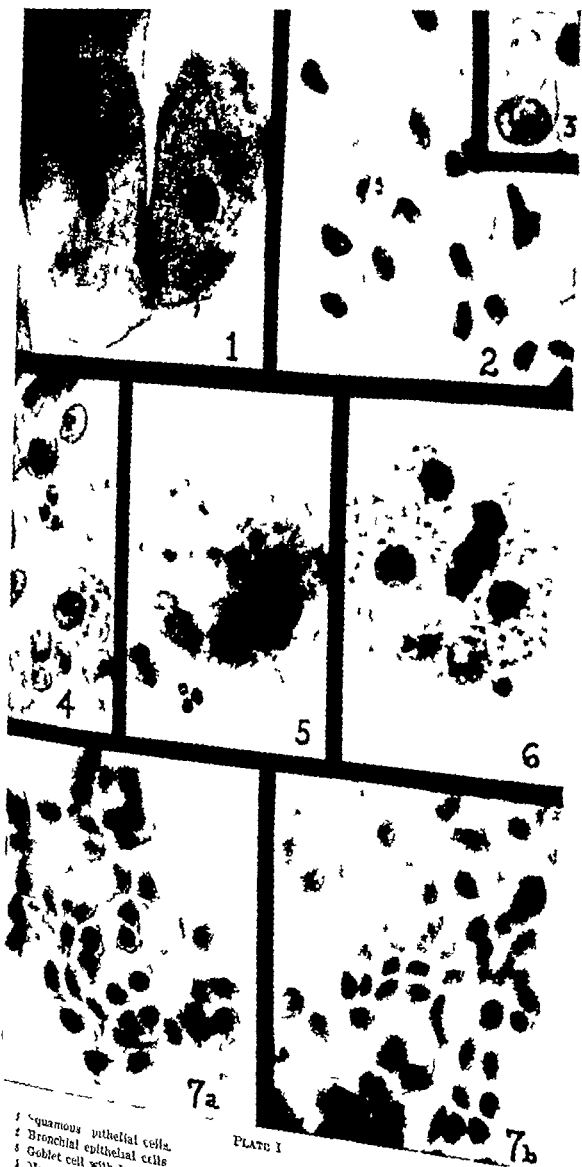


PLATE I

1 Squamous epithelial cells.

2 Bronchial epithelial cells

3 Goblet cell with large mucigen droplet in the cytoplasm.

4 Macrophages, some with ingested particles.

5 Giant multinucleated macrophage with cytoplasmic vacuole. Note neutrophil for comparative size.

6 Giant multinucleated macrophage with numerous ingested particles.

7 a and b Squamous metaplasia of bronchial epithelium with degenerative changes.

From cases of bronchiectasis

All photographs were taken under oil immersion lens (X130) except where noted

*Cells From the Blood*—The erythrocytes and leucocytes encountered in the sputum are as easily identifiable as in the usual tissue sections. Occasionally, when the sputum is purulent, the number of leucocytes is so great as to obscure the other cellular elements. In these instances it has been our policy to recommend a course of penicillin therapy to reduce the number of inflammatory cells and thereby facilitate proper sputum examination.

*Cells From the Mouth and Pharynx*—(Plate I, 1) These cells arise from squamous epithelium and are large and polygonal in shape. They contain an abundant amount of pale pink cytoplasm, with small centrally located nuclei. The nucleus is usually light blue, round to oval in shape, and surrounded by a distinct nuclear membrane. It has a finely granular chromatin network and may have a small distinct nucleolus. The cytoplasm may contain numerous invading bacteria. These squamous epithelial cells may occur singly or in sheets. The number of these cells present is a good index of the amount of saliva contained in the sputum. Their identification offers no difficulty.

*Cells From the Epithelium of the Tracheobronchial Tree*—(Plate I, 2 and 3) The tall ciliated columnar cells which line the trachea and bronchi are frequently present in the sputum and may occur singly or in clumps. The basal end may be drawn out into a long filamentous process. The nucleus is basally located, homogeneous in appearance, and stains deep blue. As many as three nuclei have been observed in a single cell. When these columnar cells occur singly and are not influenced by the presence of surrounding cells, they may assume a spherical shape. The ciliated border can be made more distinct by decreasing the aperture of the substage condenser. Goblet cells may be seen in the sputum. They can be recognized by the presence of a large mucigen droplet in the distal part of the cell, as seen in Plate I, 3.

*Macrophages*—(Plate I, 4, 5, 6) These are large round cells which may occur singly or as clusters of individual cells and are found in almost all sputa. They contain moderate-sized nuclei and abundant cytoplasm. The nuclei may be round, oval, or bilobed and usually have small nucleoli. However, at times the nucleolus is quite prominent. The chromatin is rather evenly distributed throughout the nucleus. Occasionally multinucleated giant macrophages are seen. The cytoplasm may be clear or may contain a variety of ingested particles and vacuoles. The blood pigment-containing cells (*heart failure cells*) have been found to be associated not only with chronic passive congestion of the lungs, but may also be found in a variety of conditions, especially those in which hemoptysis has occurred.

#### NEOPLASTIC CELLS

There are certain characteristics which most cancer cells have in common, regardless of the type of tumor from which they arise. These are as follows: (1) Neoplastic cells tend to occur in groups or tumor fragments. (2) Marked hyperchromasia of the entire cell is an outstanding characteristic and serves to make the malignant cells conspicuous even under low power magnification. (3) There is complete loss of polarity in the arrangement of neoplastic cells in a tumor fragment. (4) There usually is marked variation in the size and shape of malignant cells and then nuclei in any one tumor. (5) The nuclei of tumor

cells are generally large relative to the size of the cell. Multiple nuclei are not uncommon. (6) The nucleoli are large, distinct, often multiple, and may take a pink stain in contrast to the dark blue color of the nuclei. (7) Malignant cells may be phagocytic and it is not unusual to find one malignant cell ingested by another (Plate IV, 18). (8) Neoplastic cells may contain large, deep blue cytoplasmic bodies, which are known as bird's eye inclusions of Leyden (Plate IV, 20). The nature of these bodies has not been established, but it is thought that they probably arise from the multiplication of centrosomes, or that they are retained secretory products of the cell.<sup>3</sup> The neoplastic cells in the sputum fall into two major categories: undifferentiated and classifiable.

1 *Undifferentiated Cells*—(Plate II, 9) These are tumor cells which possess only the described general characteristics. They arise from a totally undifferentiated tumor or from undifferentiated portions known to occur commonly in squamous carcinoma, adenocarcinoma and small cell carcinoma. Therefore undifferentiated neoplastic cells in the sputum may or may not reflect the predominating histologic character of a tumor. Conversely, a histologically differentiated tumor can give rise to both undifferentiated and classifiable elements in the sputum.

2 *Classifiable Cells*—These cells, in addition to the general characteristics of neoplastic cells, have certain distinguishing features which allow one to classify them as to the type of tumor from which they arise. (a) *Squamous carcinoma cells* (Plate II, 10, and Plate III, 11, 12) The squamous carcinoma cells tend to be elongated in shape. The nuclei are usually oval to spindle shaped, but may be round and have a dense, coarsely granular chromatin structure. Nucleoli may be single or multiple and are usually very prominent, large and pink staining. The two characteristics that are diagnostic of this type of tumor are keratinization of the cytoplasm which imparts to it a deep pink to red color, and the arrangement of the tumor cells in whorls. True epithelial pearls are not uncommon. (b) *Adenocarcinoma cells* (Plate III, 13, 14) Cells of this type are usually rather large and round or oval in shape. The nuclei are round, frequently multiple and commonly contain one or more small discrete nucleoli. Occasionally single large nucleoli are seen. The cytoplasm is usually abundant and several small or one or two large clear droplets of secretion are present. Secretory vacuoles are the only valid criterion for the diagnosis of adenocarcinoma unless one finds malignant cells forming true acini. This arrangement, however, is quite rare. (c) *Small cell carcinoma* (Plate III, 15) Small cell carcinoma cells are the smallest of the malignant cells in the sputum. They are round or elongated in shape and of fairly uniform size. The nucleus fills the cell almost completely so that only a narrow rim of cytoplasm is detectable. The nucleus is finely granular, moderate-sized single nucleoli are the rule. To the untrained eye these cells resemble large lymphocytes except that they occur as fragments of tissue.

#### DISCUSSION

In applying the described criteria for malignant cells to the examination of sputum, one must be aware of certain pitfalls which may cause erroneous diagnoses. Overstaining with hematoxylin may so obscure the structure of the

nuclei as to render adequate study difficult and to make it impossible to appreciate hyperchromasia, because all the nuclei, benign or malignant, will appear dark blue. One of the important criteria of malignancy is thus lost.

Another possible source of error may be the presence of degenerating normal cells. The changes brought about by this process include hyperchromasia and swelling of the nucleus, vacuolization of the cytoplasm, and changes in size and shape of the cell. Degenerating normal cells may thus assume what appear to be characteristics of malignancy. But careful observation will disclose loss of distinct cellular and nuclear outlines and loss of nuclear detail. Frequently, in the close vicinity one finds similar but better preserved benign cells, and others which are obviously and more completely degenerated. Occasionally macrophages which have no visible ingested material in their cytoplasm and whose nucleoli are rather prominent are encountered. Such cells may simulate undifferentiated neoplastic cells, or, if they contain vacuoles, cells arising from adenocarcinoma. However, phagocytes occur only as individual cells and are not hyperchromatic, and similar cells with ingested material can usually be found in the immediate vicinity.

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Occasionally, one is tempted to make a positive diagnosis on the basis of one or two single cells which have all the characteristics of malignancy. It is wise in such instances to withhold the final diagnosis until further specimens can be examined. In almost every case one will eventually find either numerous other similar cells or small fragments of tumor tissue. It has been shown that the examination of three separate sputum specimens will yield about 96 per cent of the positive diagnoses.<sup>9</sup> However, if the clinical history and roentgenographic studies strongly indicate malignancy, and especially when bronchoscopic biopsy is impossible, as in upper lobe lesions,<sup>1</sup> it is often desirable and worth while to repeat the examination several more times.

The diagnosis given to the clinician should be concise and explicit. We feel that the grouping of cells as to the probability of malignancy is confusing and even dangerous. Unless the pathologist is absolutely certain that carcinoma cells are present in the sputum, he should render a negative report. Care should be taken to advise the clinician as to the significance of positive and negative reports. The presence of cancer cells in the sputum is not a priori evidence of primary bronchogenic carcinoma. The cells may arise from carcinomas of the mouth, pharynx, nasopharynx, larynx, esophagus, trachea, or from pulmonary metastases, and in such instances cannot be differentiated from cells of primary bronchogenic neoplasms. Above all, one must be aware of the fact that failure

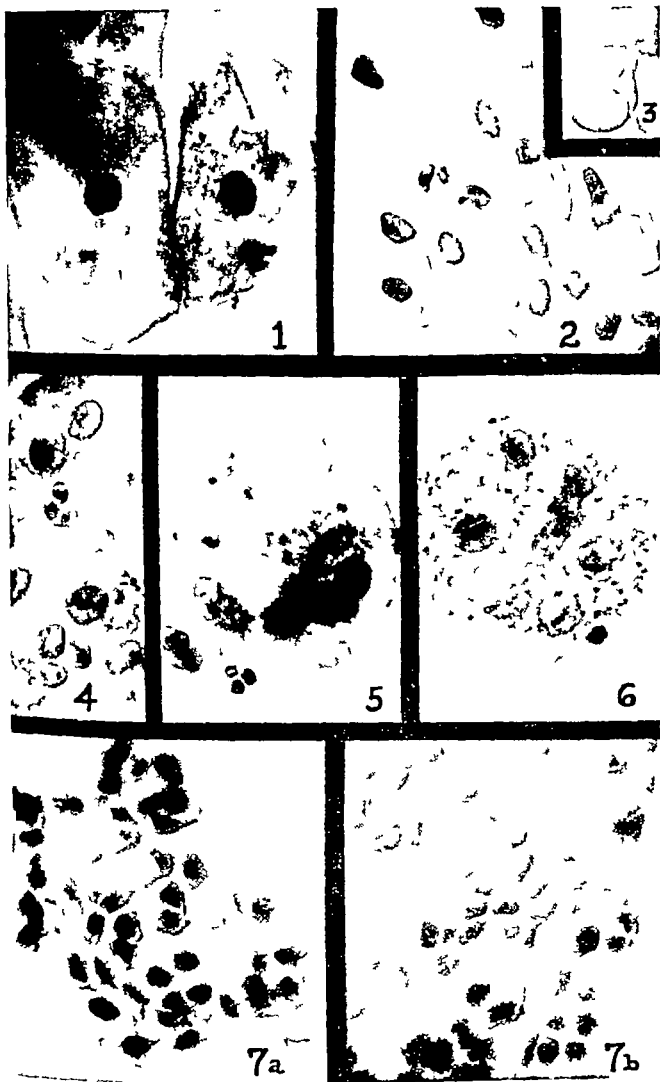


PLATE I

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- 5 Giant multinucleated macrophage with cytoplasmic vacuole Note neutrophile for comparative size
- 6 Giant multinucleated macrophage with numerous ingested particles
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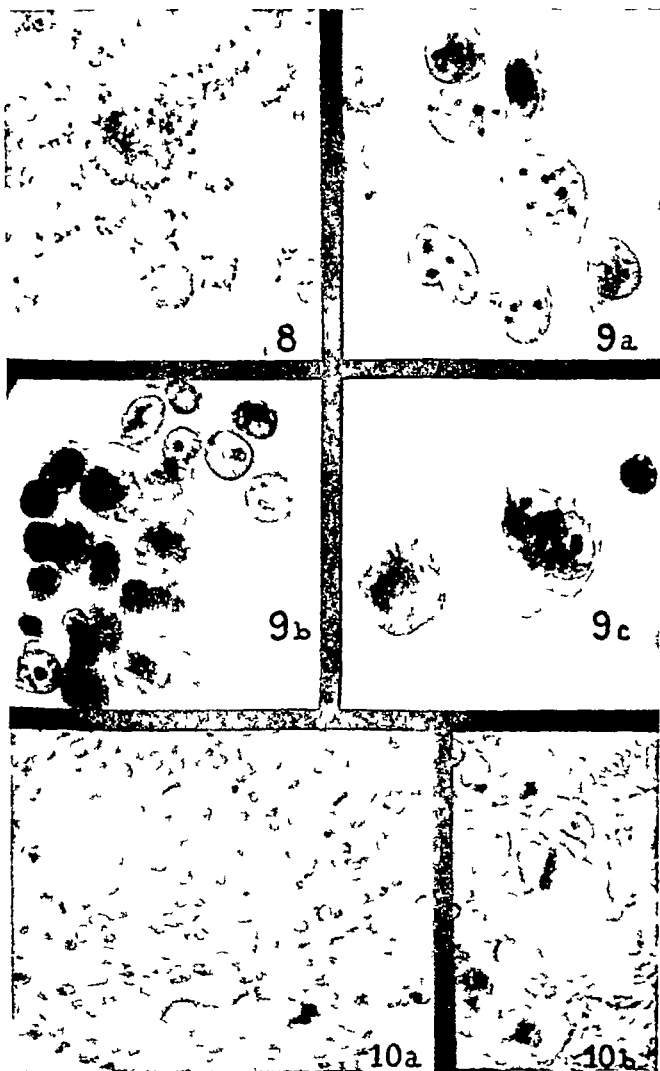


PLATE II

8 Conspicuous hyperchromatic tumor fragments as seen under low power magnification ( $\times 140$ )

9 Undifferentiated cells from (a) Undifferentiated embryonal carcinoma  
(b) Squamous carcinoma.  
(c) Adenocarcinoma.

10 Squamous carcinoma. (a) Large fragment of tumor (high-dry magnification ( $\times 610$ ))  
(b) Mitotic figure.



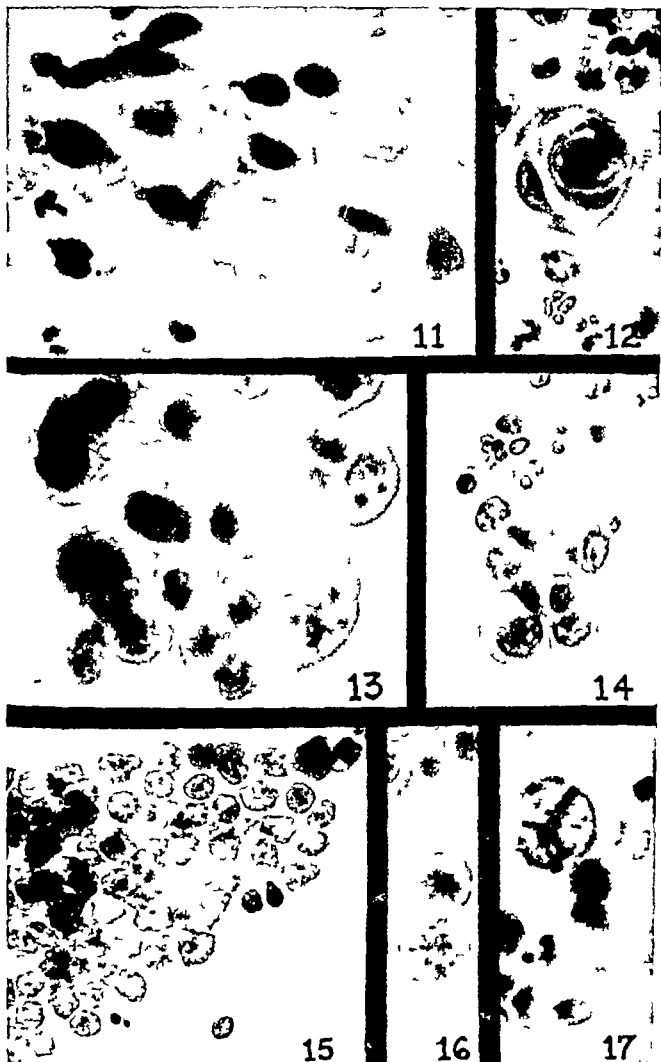


PLATE III

- 11 Squamous carcinoma Note elongated large tumor cells
- 12 Squamous carcinoma Epithelial pearl
- 13 Adenocarcinoma Note multinucleated cells and vacuolated cytoplasm
- 14 Adenocarcinoma Cells with secretory vacuoles
- 15 Small cell carcinoma Note resemblance to lymphocytes
- 16 Undifferentiated carcinoma Two mitotic figures
- 17 Squamous carcinoma Atypical nuclear division

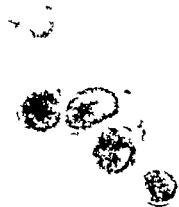




18



19



20



21a



21b



21c

PLATE IV

- 18 Adenocarcinoma. One tumor cell phagocytized by another.  
 19 Adenocarcinoma. Tumor cells phagocytized by large macrophage. Note pale nucleus of macrophage just below upper group of ingested malignant cells.  
 20 Adenocarcinoma. Bird's eye inclusion.  
 21 a, b and c Squamous carcinoma. Unusual configurations of tumor cells.



to find malignant cells in the sputum does not rule out carcinoma and should be disregarded if the weight of other clinical or radiologic evidence favors this diagnosis

## CONCLUSION

Easily recognizable malignant cells occur in the sputa of more than 70 per cent of cases of pulmonary cancer. When one restricts positive diagnoses to these comparatively obvious cells the danger of false positive diagnoses is reduced to a minimum while at the same time the test retains a good degree of accuracy. With the realization of these facts sputum examination becomes more suitable for widespread adoption as a routine laboratory procedure.

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## A MICROADAPTER FOR THE EVELYN MACROCOLORIMETER

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MINNEAPOLIS, MINN.

THE Evelyn macrocolorimeter<sup>1</sup> requires the use of a minimum volume of 6 c.c. of solution in order to obtain a satisfactory colorimetric reading. It would be advantageous in some clinical procedures, such as the Evans blue dye blood volume method, to obtain a reading on a much smaller volume. The Evelyn microcolorimeter,<sup>2</sup> which allows colorimetric readings with volumes of solution ranging from 0.1 to 2.0 c.c. is technically difficult to operate, therefore, its application to routine laboratory procedures is limited.

In order to obviate the inherent difficulties of the Evelyn microcolorimeter, the microadapter described here was designed to permit readings on volumes as low as 1 c.c. with the Evelyn macrocolorimeter. Flat-bottomed microcolorimeter tubes,\* 10.8 mm. in inside diameter, can be used with the adapter, in the Evelyn macroinstrument.

*Construction*—The adapter, shown in Fig. 1, is constructed of bakelite. The outside diameter is 22 mm. which permits easy removal from and insertion into the bakelite tube sleeve of the Evelyn. The projection on the bottom of the adapter fits into a slot cut in the inside bottom of the tube sleeve and locks the adapter in place with its aperture opposing the 6 c.c. aperture of the tube sleeve.

The aperture of the adapter is cut slightly larger than that of the tube sleeve, so that the sleeve aperture determines the light path. The inside diameter of the adapter, 12.3 mm., was chosen to accommodate the microtubes. The cut-outs along the side of the adapter permit the operator to guide the tube, which is  $4\frac{1}{2}$  inches in length, as it is put into or taken from the adapter. The adapter has an over-all length of 7 and  $7/16$  inches including the  $3/16$  inch projection.

*Performance*—The microtubes are calibrated for equality of transmission in the same manner as Evelyn tubes. To obtain a center setting the adapter need be only partially removed until clear of the light beam through the sleeve aperture, this maneuver permits rapid checks of both center settings and readings.

Readings were made with the Evelyn macrocolorimeter with and without the adapter, using the same Evans blue solutions in serial concentrations from 0.0015 mg. per cubic centimeter to 0.0105 mg. per cubic centimeter. One series of results is presented in Table I. The "L values" (photometric density) obtained with the adapter were multiplied by 19.5/10.8 (the ratio of the diameters of the Evelyn and microtubes) in order to compare the two sets of readings. It may be noted that density values obtained with the adapter, when corrected in this manner for the difference in diameter, agree quite closely with those obtained without the adapter.

From the Laboratories of the University of Minnesota Hospitals.

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\*Available from Hellige Inc. Long Island N. Y. under the name Hellige Diller Micro Tubes.





Fig. 1—An oblique view of the adapter

TABLE I COMPARISON OF EVELYN MACROCOLORIMETER READINGS WITH AND WITHOUT ADAPTER

CONCENTRATION OF DYE SOLUTION (MG /C C )	READING WITHOUT ADAPTER	L VALUE WITHOUT ADAPTER	READING WITH ADAPTER	L VALUE WITH ADAPTER	L VALUE WITH ADAPTER CORRECTED FOR DIFFERENCE IN DIAMETER OF TUBES
0015	85 <sup>3</sup>	0667	91 <sup>3</sup>	0374	0675
0030	74 <sup>3</sup>	1278	85	0706	1275
0045	66 <sup>2</sup>	1772	79 <sup>3</sup>	0982	1772
0060	57 <sup>2</sup>	2384	73	1367	2434
0075	51	2924	67 <sup>3</sup>	1691	305
0090	45	347	64	1922	347
0105	40	398	59	2291	412

In addition, for any given concentration of Evans blue solution, the adapter gave the same reading with a volume of 1, 2, or 5 cubic centimeters. It appears that use of the adapter successfully increases the volume range of the Evelyn macrocolorimeter from 6 c c down to 1 cubic centimeter.

## SUMMARY

A microadapter for the Evelyn macrocolorimeter has been described which permits readings on volumes of solution as low as 1 cubic centimeter. Evidence has been presented to substantiate the validity of results obtained with the adapter.

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## VENOUS CATHETERIZATION WITH POLYETHYLENE TUBING

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PHILADELPHIA, PA.

THE use of polyethylene tubing in the catheterization of veins has been reported by Zimmermann<sup>1</sup> and Meyers.<sup>2</sup> It is the purpose of this communication to describe and evaluate a method of taking samples of portal vein blood in the ambulatory dog, and to record some of the observations made on the animal reaction to the plastic polyethylene ‡

### TECHNIQUE

Under general anesthesia with 1 per cent Nembutal a transverse right subcostal muscle splitting gridiron incision is made. The peritoneum is dissected free by blunt dissection and reflected medially. The portal vein is approached retroperitoneally and is then followed cephalad toward the hepatic border. The peritoneum is frequently opened just as the portal vein is approached. A site is selected just cephalad to the mesenteric radicals in the main trunk of the vein. The loose areolar tissue is dissected away exposing the vein wall. A purse string suture of fine black silk is placed but not tied in the vein wall. Then a No. 16 gauge needle is thrust through the center of the purse string suture, with the end of a small caliber polyethylene tube about 12 inches long inside the needle bore but not projecting beyond the needle bevel (Fig. 1). Tubing with an internal diameter of 0.023 inch has been found most satisfactory. After piercing the vein wall the cannula is pushed through the needle bore into the lumen of the vein for about 3 centimeters. The needle is then pulled out of the vein and the polyethylene tubing is held stationary by a pair of thumb forceps. As the needle is pulled clear of the plastic tubing the purse string suture is tied. The plastic tubing is then aspirated. If aspiration is adequate several loops of the suture are placed around the tubing, slipped down next to the vein wall and tied again. Sterile saline is injected into the tubing and the end of the tubing sealed by heating the tip of the tubing and then pinching it with forceps while still hot. The same procedure may be used for inferior vena cava catheterization.

We have used as a criterion of an adequate catheterization the aspiration of 5 c.c. of blood in thirty seconds.

The tubing is brought out retroperitoneally through a separate stab wound and the incision closed. The tubing external to the skin is coiled and is held in place with a piece of gauze saturated with collodion. A firm body encircling adhesive dressing is then applied.

### RESULTS

We have performed twenty-four portal vein catheterizations in twenty dogs. The first attempt at aspiration of the vein catheter usually was made seven days after insertion. In four no blood could be aspirated on the first or subsequent trials. In eleven either the animal pulled the tubing out or the tubing fell out or the dog died before aspiration was attempted. A number of the failures could

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‡The polyethylene used in these experiments was purchased from the Surprenant Electrical Insulating Company, Boston, Mass. It was furnished us as pure polyethylene without the addition of a plasticizer.

be attributed to preventable errors in technique. The remaining nine catheterizations functioned well for an average of twenty-one days after catheterization. The maximum time adequate blood samples were obtained from the portal vein was thirty-four days. In several animals the portal vein was successfully catheterized two or more times at intervals of several weeks. In only one animal did

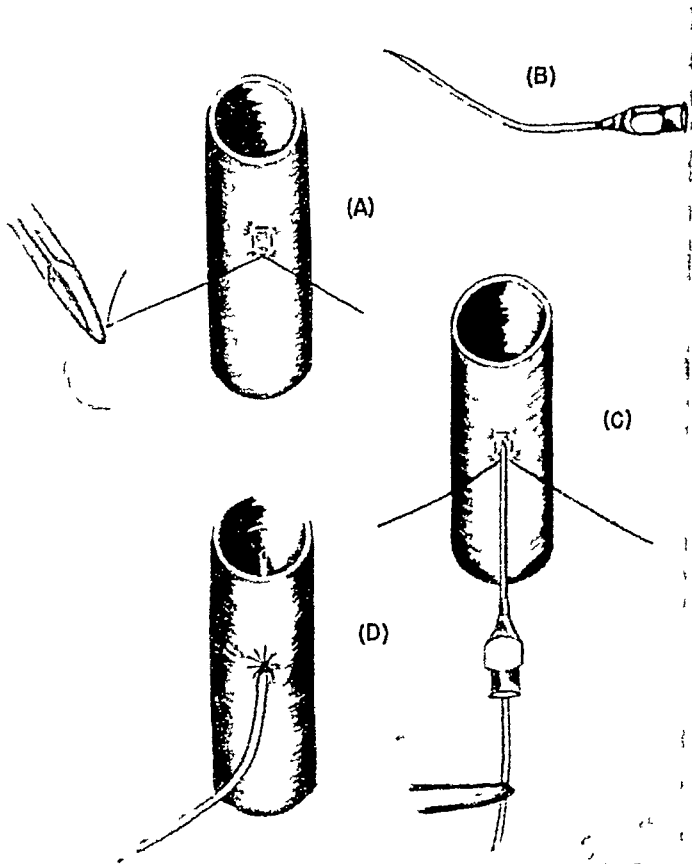


Fig 1—Technique of insertion of fine polyethylene tubing into portal vein lumen.

complete thrombosis of the portal vein result, and this followed three separate catheterizations of the portal vein at two- to three-week intervals, the last tube having been left in the vein for two months.

Although adequate specimens of blood were aspirated for prolonged periods in 37.5 per cent of the experiments, some degree of reaction to the polyethylene tubing was noted in every case where the portal vein was examined.

The first reaction to the polyethylene tubing was the formation of a thrombus surrounding the intraluminal portion of the tubing (Fig 2). Later, fibrous tissue was laid down over the tube with puckering of the adjacent intimal lining (Fig 3). These events took place in from two to four weeks after the tubing was inserted into the vein lumen. In one instance the tubing was completely excluded from the vein lumen by fibrous tissue at three weeks. The tip of the tubing remained free in most cases, however, although an area of granulation

was sometimes seen on the intima adjacent to the free tip of the tubing. Attempts to obtain blood samples over periods of several days from veins or small size catheterized with polyethylene tubing were unsuccessful. It is evident from Figs 2 and 3 that the lumen of a small vein may become occluded promptly.

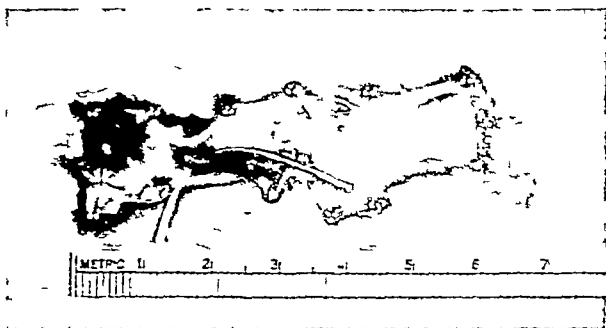


Fig. 2.—Polyethylene tubing inserted into portal vein four days prior to sacrifice of the dog. Note the thrombus formation surrounding the intraluminal portion of the tubing.

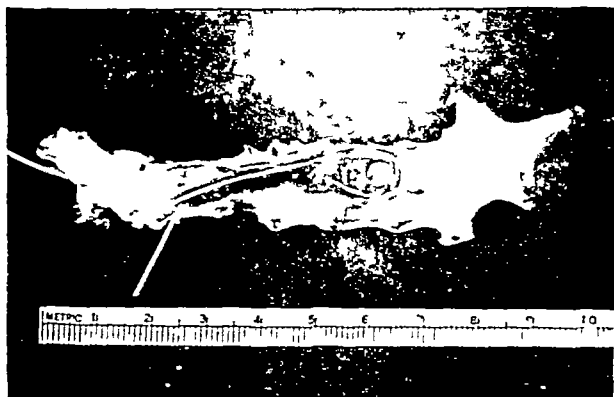


Fig. 3.—Polyethylene tubing inserted into portal vein twenty-two days prior to sacrifice of the dog. Note coiling of excess tubing; arrow points to open end. Scarring and puckering of intimal lining around polyethylene tubing. Samples of portal blood were aspirated through tubing for fifteen days.

#### DISCUSSION

It is apparent from these experiments that catheterization of large veins may be carried out with polyethylene tubing of small caliber and that satisfactory blood samples may be obtained over a period of approximately three weeks.

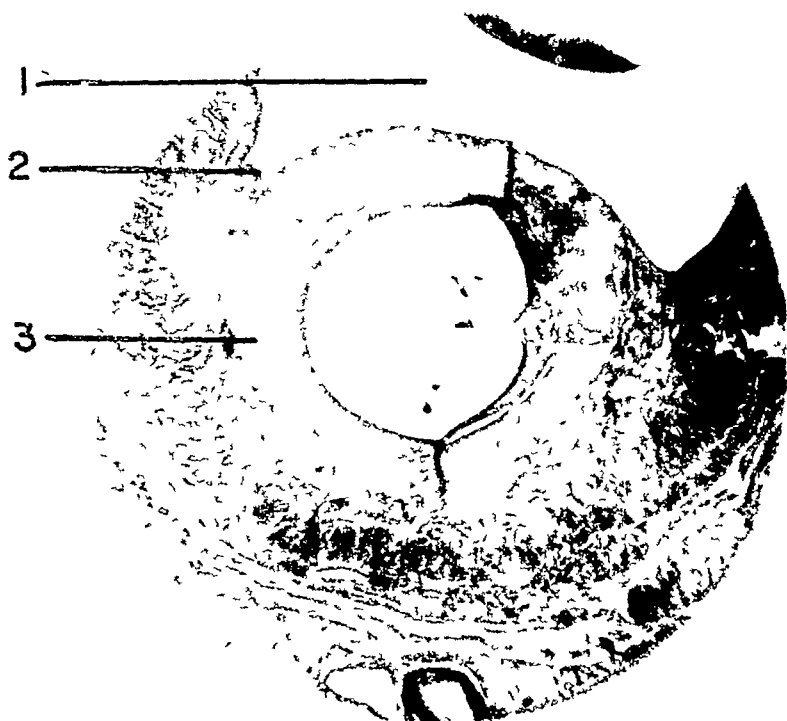


Fig 4A —Microphotograph of portal vein  $\times 16$  Polyethylene tube inserted into vein twenty days prior to sacrifice of dog 1, Lumen of vein 2, Junction of intimal surface of vein wall and tissue overlying polyethylene tube See Fig 4B 3 Fibrous tissue surrounding space where polyethylene tube lay Tube dislodged or dissolved during preparation of histologic section

Repeated catheterizations are possible although there is evidence that increasing damage to the vein wall results. The use of polyethylene tubing is an improvement over the London cannula<sup>3</sup> for larger vessels but is not satisfactory for vessels of small size.

There has been some controversy over the tissue reaction to polyethylene. Ingraham and co-workers<sup>4</sup> emphasized the slight tissue reaction to this plastic in the pure form. They believed that the marked fibrous tissue response noted by Poppe and de Oliveira<sup>5</sup> to sheets of polyethylene was due to the presence of a plasticizer. For this reason Ingraham and associates<sup>4</sup> insisted on *pure* polyethylene to which they found only slight reaction when it was buried in cerebral tissue of various experimental animals. Zimmermann<sup>1</sup> did not indicate whether he used polyethylene in the *pure* form, but it is of interest that in two of the four experiments where the veins were examined, thrombosis was found. He was not certain whether this reaction was due to the tubing *per se* or to the infused fluid. There was no infusion of fluids in any of the experiments recorded here except the small amount used to flush the tubing out at the beginning and end of each aspiration. The reaction to pure polyethylene when placed in the lumen of a blood vessel may, however, differ from the reaction in soft tissues.

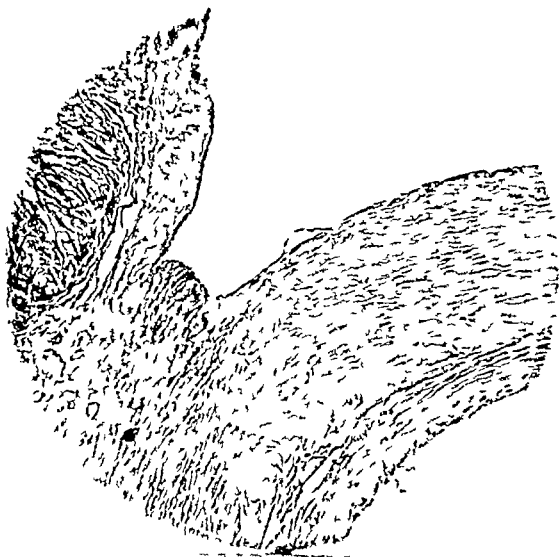


Fig 4B—Microphotograph of junction of vein wall and fibrous tissue surrounding polyethylene tube. Tub. is no longer present. ( $\times 100$ )

due to the formation and subsequent organization of a blood clot surrounding the tubing. It was our impression that the fibrous tissue reaction to the tubing was progressive and that the longer the plastic remained in the lumen of the vessel the more pronounced the reaction became (Figs 4A and 4B). This suggests the possibility of using this material intraluminally for the gradual occlusion of arteries.

#### SUMMARY

The catheterization of the portal vein for the purpose of obtaining blood samples from the ambulatory dog over prolonged periods can be satisfactorily performed with small caliber polyethylene tubing. The retroperitoneal approach to the portal vein lessens the period of postoperative discomfort and renders the animal available for study at an earlier time. The inferior vena cava is exposed simultaneously by this approach and may also be catheterized.

The presence of pure polyethylene in the lumen of a vein results in a fibrous tissue response that tends to exclude the tubing from the lumen of the vessel.

with narrowing of the lumen of the vein. It is not clear how much of the fibrous tissue reaction is due to the organization of the thrombus that surrounds the tubing. The fact that the fibrous tissue reaction appears to be progressive, however, would seem to indicate that polyethylene per se possesses some irritative action in blood vessels.

The authors wish to acknowledge the helpful advice of Dr C. Martin Rhode and Dr William M. Parkins of the Harrison Department of Surgical Research, University of Pennsylvania.

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## SCRLW SYRINGE\*

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A METHOD for carrying out microcolorimetric capillary tube analysis has been described using pipettes similar in construction to the white blood cell counting pipettes<sup>1</sup>. The screw syringe was devised for more precise control of the flow of fluid within the capillary tubes. This screw syringe also can be used to improve the accuracy of diluting blood for cell counts, for controlling the flow of liquids in micropipettes (0.01 to 1 cc) and for controlling the flow of radioactive and other toxic agents within ordinary pipettes thus avoiding the danger of sucking noxious materials into the mouth.

### CONSTRUCTION

The syringe consists of the following parts (Figs. 1 and 2): A rubber collar (*A*) contained within the collet (*B*) fixes the pipette firmly in place when the collet is tightened. The body of the control device (*D*) contains the plunger (*F*) as well as the screw control (*E*) for this plunger. Two doughnut shaped 'O' rings (*I* and *J*) seal the plunger within the body (*D*) and within the screw control (*F*). Because 'O' ring (*J*) is compressed more than is 'O' ring (*I*) the plunger (*F*) will turn with the control screw (*E*) upon rotating the latter and this provides the delicate control of the liquid within the pipette. However the plunger can be withdrawn directly from the body without the use of the control screw (*E*) and thus provides a coarse adjustment for the fluid volume. An air vent avoids the displacement of the liquid within the pipette when the latter is removed from the collet. The 'O' ring (*H*) is placed within the body of the syringe and seals the vent hole (*G*) when the collar (*C*) is loose. When the collar (*C*) is tightened the 'O' ring is distorted the hole in the body (*D*) is uncovered and the vent is open. The capacity of the screw syringe is 3 cubic centimeters.

### USE OF CONTROL DEVICE FOR BLOOD COUNTING AND BLOOD CHEMISTRY MICROPIPETTES

The collet, containing an appropriate rubber adapter is attached to the syringe body, the pipette is inserted and fixed in place by tightening the collet. The air vent is closed by loosening the collar (*C*), and the apparatus is ready for use. By means of control screw (*E*, Fig. 3) a drop of blood is sucked into the stem of the pipette slightly above the mark, the excess blood is wiped off the tip of the pipette and the blood is adjusted precisely to the mark by simultaneously rotating the control screw and wiping the tip of the pipette on the operator's finger. The tip of the pipette is then placed in diluting fluid (Fig. 4) which is sucked into the bulb of the pipette by withdrawing the plunger (*F*). As soon as the meniscus approaches the upper capillary tube of the pipette, the screw control (*E*) is used to accomplish the final exact adjustment. The pipette is re-

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The screw syringe is available from the Micrometric Instrument Company, Cleveland

Ohio. The use of the differential 'O' ring pressure was suggested by A. Howard of the Micrometric Instrument Company.

moved from the diluting fluid and the tip carefully wiped with the finger or Kleenex (If Kleenex is used, one must avoid placing it against the tip of the capillary in order to avoid withdrawal of fluid by capillary action)

The index finger of the left hand is placed over the tip of the pipette, and while holding the bulb of the pipette between the thumb and middle finger, the body of the screw syringe is rotated with the right hand, and the pipette is

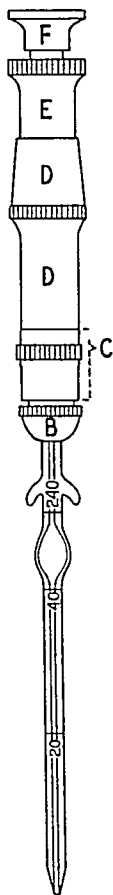


Fig 1

Fig 1 —Screw syringe with micropipette inserted

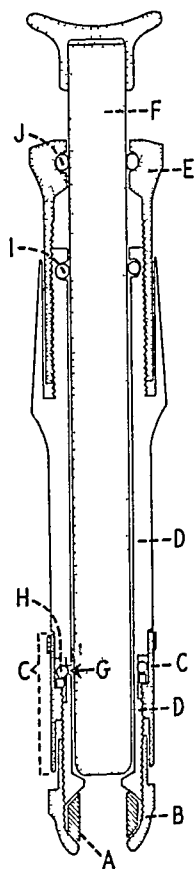


Fig 2

Fig 2 —Construction of screw syringe

removed. The finger seal prevents the displacement of the fluid within the capillary during removal. An alternate method for removing the pipette from the screw syringe utilizes the vent. The pipette and its holder are placed in a near horizontal position, the air vent is opened, the collet is loosened, and the pipette is removed. (It placed in an absolute horizontal position, some pipettes show a slight upward movement of the contained liquid. The upward displacement is due to the fact that adjustment to the upper mark was made in the vertical position against the hydrostatic pressure of the liquid in the pipette, whereas in the horizontal position this pressure no longer exists. However, if the pipette is held at a slight angle, 5 to 20 degrees to the horizontal, the liquid will not rise

above the mark nor will it run out of the pipette when the vent is opened.) Thus all can be accomplished without in any way affecting the position of the meniscus within the micropipette.

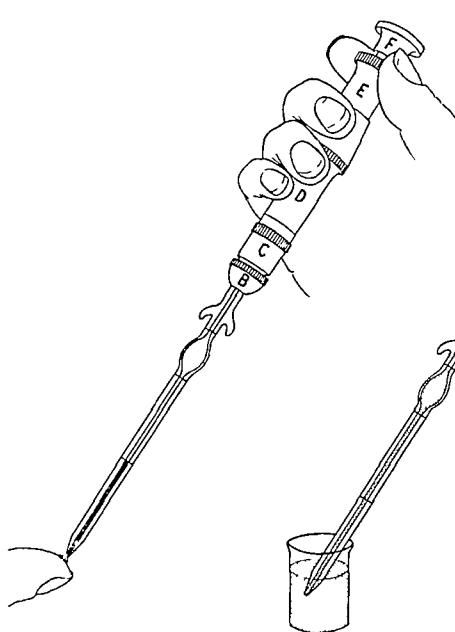


Fig 3

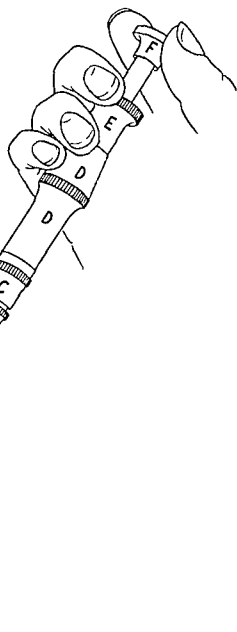


Fig 4

Fig 3—Measuring a drop of blood in the micropipette with the fine control crew (E)  
 Fig 4—Diluting the blood in the bulb of the pipette using the plunger (F)

#### USE OF SCREW SYRINGE WITH 1 OR 2 C C PIPETTES FOR RADIOACTIVE OR OTHER TOXIC LIQUIDS

The pipette is inserted into the collet and the vent is closed. By means of the plunger (F) the liquid is sucked up into the pipette above the desired mark. The outside of the pipette may be wiped if desired, the tip of the pipette is then placed against the side of the bottle and the final adjustment to the mark is made with the screw device. The pipette is transferred to the desired container, the vent is opened and the pipette allowed to drain by gravity.

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# PROCEEDINGS OF THE CENTRAL SOCIETY FOR CLINICAL RESEARCH

Twenty-First Annual Meeting

Chicago, Ill., Oct 29 and 30, 1948

## ADDITIONAL ABSTRACTS—Concluded

### 69 THE CRYSTALLIZATION OF SEROTONIN

IRVING H. PAGE, M.D., MAURICE M. RAPPORT, M.D. (BY INVITATION), AND  
ARDA ALDEN GREEN, M.D. (BY INVITATION)  
CLEVELAND, OHIO

When organs are perfused with blood or serum, progressive vasoconstriction often makes it all but impossible to force blood through them. The substance causing this vasoconstriction has never been identified nor isolated. In view of its probable importance in the vasoconstriction occurring after rupture of thrombosis of vessels such as the coronaries, or after tissue injury in shock, it seemed important to find out something of its chemical nature.

The isolation from beef serum depended on (1) precipitation of beef serum proteins with alcohol, (2) precipitation of salts, phosphatides, and amino acids with acetone, (3) chloroform extraction, (4) extraction of the active principle with butyl alcohol, (5) precipitation of the active principle from butyl alcohol with 5-nitrobarbituric acid, (6) decomposition by addition of acetone to hot aqueous solution, (7) extraction of filtrate residue with warm absolute methanol, (8) recrystallization from water-acetone.

Thin, rhomboid plates (m.p. 212-214°) were obtained which seem homogeneous. Color reactions and ultraviolet absorption indicate the presence of an indole nucleus in the structure. The empirical formula is  $C_{11}H_{23}O_3N_2S$ . Ionic sulfate analysis suggests that Serotonin is a sulfonic acid salt of an organic base with the formula  $C_{11}H_{21}O_3N_2 \cdot H_2SO_4$ .

Serotonin is more than twice as active as adrenalin in causing constriction of the vessels of perfused rabbit ears. Isolated strips of rabbit ileum are contracted by it. Injected intravenously into dogs or cats it caused a sharp rise in arterial pressure much like that of adrenalin. The rise was augmented by sympathectomy. It does not appear to produce tachyphylaxis when given repeatedly in small doses.

An enzyme has been prepared from lung which inactivates Serotonin.

### 70 MANAGEMENT AND CLINICAL COURSE OF LOWER NEPHRON NEPHROSIS

WILLIAM S. HOFFMAN, M.D., AND DANIEL MARSHALL, M.D. (BY INVITATION)  
CHICAGO, ILL.

Six patients with lower nephron nephrosis from a variety of causes have been managed by a regimen that included a deliberate, slow induction of edema of nearly normal electrolyte composition for the purpose of diluting the accumulating intoxication products. Other measures were designed to keep the patient in a good state of nutrition. The aim was to keep the patients from dying in uremia before the renal lesion began to subside. Five out of the six patients recovered, the sixth died in uremia and heart failure five days after the onset of diuresis. Four of the recovered patients had complete restoration

to normal renal function. One patient, a child of 2 years, still had moderate renal insufficiency five months after the onset of diuresis. This condition was associated with the development of bilateral calcinosis of the kidneys as seen on x ray.

In these patients, the oliguric phase lasted from five to thirteen days. The serum nonprotein nitrogen rose to levels of 145 to 243 mg per 100 cc and did not begin to subside until several days after diuresis began. Edema occurred early even when fluids were not specially administered. The serum chloride and sodium concentration tended to reach dangerously low levels (in one instance the chloride concentration was 58 meq per liter). This development had to be combated by infusions of 2 per cent salt solution. With careful control of the quantity and composition of the edema fluid symptoms of uremia were minimal, and the patients ate well without vomiting. The leveling of the serum non protein nitrogen concentration during the latter part of the oliguric period was due either to the diluting effect of the edema or to diminished tissue breakdown brought about by the improved nutrition.

## 71 STUDIES ON PROLONGED SUPPURATIVE INFECTION IN MAN

GEORGE W. JAMES III, M.D., LILLIAN A. RIBLET, M.D., JOSEPH C.  
ROBINSON, M.D., ROBERT E. JOHNSON, M.D., AND ROBERT M.  
KARK, M.D., CHICAGO, ILL.

(INTRODUCED BY ROBERT W. KEETON, M.D.)

Despite advances in surgical techniques and the vigorous use of blood and plasma transfusions, sulfonamide derivatives and antibiotic substances, suppuration persists in a number of patients with infected traumatic injuries. Therefore, during the past year clinical bacteriologic and laboratory studies were made on ninety one young men suffering with chronic suppurating lesions of the bones, kidneys and other soft tissues. A group of twenty seven patients and ten bedridden control subjects were studied more intensively than the remaining sixty four patients.

Although the average period of hospitalization was twenty three months at the beginning of observation fair nutritional status was maintained. There was an average weight loss of 7.9 kilograms but no clinical evidence of vitamin or protein deficiencies.

Sixty three per cent of the patients were infected with *Staphylococcus aureus*. Pathogenic spore bearing organisms *Proteobacterium aerogenes*, *Aerobacter aerogenes*, and gamma (nonhemolytic) streptococci were frequently isolated. Multiple infections and concurrent self contamination were common findings, and a correlation was found between hemoglobin levels and numbers of different species of organisms in the wound. The more different genera of organisms the lower the hemoglobin levels.

Hematologic data were essentially normal. Anemia was a rare finding. Blood and plasma volumes were increased 10 to 20 per cent when expressed as a function of body weight. Total circulating hemoglobin was significantly reduced in those patients with severe infections. A marked predominance of small spherocytic reticulocytes was observed, but no other aberrant cells were seen.

Depression in serum iron and elevation of serum copper levels were more marked in patients with severe infection. Serial studies during spontaneous remissions indicate an initial shift of serum copper levels toward normal. This is followed at a later date by elevation of serum iron levels. Preliminary observations following daily oral administration of 60 mg. cobalt chloride reveal a reticulocytosis during the third to fifth week of therapy and increases in red blood cell count, hemoglobin levels, hematocrit, and total circulating hemoglobin.

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CHICAGO, ILL.

Six patients with lower nephron nephrosis from a variety of causes have been managed by a regimen that included a deliberate, slow induction of edema of nearly normal electrolyte composition for the purpose of diluting the accumulating intoxication products. Other measures were designed to keep the patient in a good state of nutrition. The aim was to keep the patients from dying in uremia before the renal lesion began to subside. Five out of the six patients recovered, the sixth died in uremia and heart failure five days after the onset of diuresis. Four of the recovered patients had complete restoration

complex possesses antibiotic activity. Normal human subjects were given crystalline penicillin G following which a 500 ml blood sample was fractionated into its various protein fractions. The penicillin was almost quantitatively recoverable in fraction VI, which in retentivity represents that portion remaining after all other fractions have been precipitated. The plasma control assays for penicillin and the fraction VI assays were identical in each instance. Additional studies were carried out by dialysis of whole plasma and some of the various fractions under temperature conditions that would not be deleterious to penicillin. There was no evidence of inactivation or antagonism of the penicillin by any of the protein fractions nor evidence of any combination with the protein fractions in the dialysis experiments. Similar studies under way with bacitracin seem analogous.

#### 74 THE ANESTHETIC AND ANTIHISTAMINIC ACTION OF A SERIES OF ANTIHISTAMINIC DRUGS IN HUMAN SKIN

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The anesthetic potency and the antihistaminic action of equimolar concentrations of Benadryl, Pyribenzamine, Neoantergan 3015 RP and 3277 RP were determined in the skin of normal human subjects. In descending order of anesthetic potency, the drugs ranked 3277 RP, 3015 RP, Pyribenzamine, Neoantergan, and Benadryl. In descending order of the ability of the drugs to inhibit histamine flares they were Pyribenzamine, Neoantergan, Benadryl, 3015 RP, and 3277 RP. The findings that those compounds which have the greatest local anesthetic potency are not those with the greatest antiflare effects supports the view that the flare-inhibiting property of these synthetic antihistaminic drugs is not directly dependent on their local anesthetic activity and there is thus a separation between antihistaminic and anesthetic actions.

#### 75 FAILURE OF ANTIHISTAMINIC DRUGS TO REDUCE REACTIVE HYPEREMIA IN MAN

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In the classic studies of Lewis and Grant on reactive hyperemia evidence was presented suggesting that this vasodilatation was due to the accumulation of a chemical in the region of circulatory arrest. Baisoun and Smirk reported that a substance with the biologic properties of histamine could be detected in the venous blood during reactive hyperemia in concentrations up to 0.6 gamma per cubic centimeter. This evidence together with the implication of Lewis studies has led to a tacit acceptance of the concept that reactive hyperemia is primarily due to the accumulation of histamine-like substance. The following observations, in which reactive hyperemia could not be reduced by antihistaminic drugs, seriously challenge the validity of this concept.

Reactive hyperemia was produced by the application and release of an inflatable occluding cuff placed about the thigh in seven subjects. These included normal subjects and one hypertensive patient before and after sympathectomy. Measurements of volume change and blood flow in the foot were made by means of a venous occlusion plethysmograph. Occlusions were maintained to produce manifest hyperemia (five to ten minutes) and threshold or minimal responses as well (one half to three minutes). The initial inflow after release

of occlusion was considered to reflect the dilatation produced. After a period of control observations of resting flow and reactive hyperemia, Benadryl (beta dimethylaminoethyl benzhydryl ether hydrochloride), 10 to 30 mg intravenously, or Pyribenzamine (beta-dimethyl-aminoethyl-2-pyridyl-benzyl ammonium chloride), 50 mg orally, was administered. Observations were repeated at intervals for a period of more than one hour. No diminution was detected in the circulatory response to arterial occlusion. The summarized averaged blood flows in cubic centimeters per minute per 100 cc limb volume, with the number of determinations in parentheses, are as follows:

	RESTING	REACTIVE HYPEREMIA
Before drug	2.70 (134)	8.33 (39)
After drug	2.62 (99)	8.78 (36)

There was no essential difference between different subjects or after varying durations of occlusions.

In several instances the intradermal injection of 0.1 to 1.0 gamma of histamine base demonstrated the effectiveness of the antihistaminic drug.

## 76 CLINICAL OBSERVATIONS ON HISTAMINE ADMINISTRATION DURING PREGNANCY

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The recent obstetric, physiologic, and pharmacologic literature continues to discuss the oxytocic effect of histamine in women and in animals. As recently as 1946, editorial comment in the *Obstetrical and Gynecological Survey* reminded that histamine exerts a powerful stimulating effect directly on the myometrium. An impressive array of experimental literature may be found to document this opinion. Two of the earlier studies conducted were those of Hotbauer and of Boune and Burn. The former author pointed out that uterine spasm is one of the observable responses in acute histamine poisoning in pregnant guinea pigs. Boune and Burn in 1927, utilizing intrauterine bags with manometer attachments in parturient women, demonstrated that histamine injected subcutaneously in a dose of 2.0 mg (reckoned in terms of base present) produced powerful but short-lived uterine contractions.

In the course of a prolonged program of clinical investigation and treatment of the so-called vascular diseases, allergic diseases, and certain atypical pain patterns not amenable to more usual forms of treatment, we have had occasion to administer or to prescribe the administration of histamine by the intravenous or subcutaneous route to fifteen pregnant women. In view of the apparent pharmacologic inconsistency of such therapy and because of the complete absence of any undesirable sequelae of such treatment, we wish to record our observations.

Our fifteen patients (twelve of whom had received a diagnosis of multiple sclerosis) were treated in a five-year period ending April, 1948. During this time approximately 70,000 intravenous injections of histamine were given and some 4,600 patients were seen in our laboratory. The series of pregnant patients included both primigravidae and multigravidae. Six patients received subcutaneous injections of histamine daily throughout the entire period of gestation. Intravenous injections of this drug were given in every month of pregnancy and in two instances the injections were given three times weekly throughout the entire third trimester to within three days of delivery. In thirteen of the fourteen patients who have thus far been delivered, there has been no



tendency to premature labor. Twelve of these fourteen have had their labor after the expected date of confinement.

The maximal amount of histamine given was administered to a primigravida, 23 years of age, who had multiple sclerosis. This patient received forty-five intravenous injections each of which contained 2.75 mg. of histamine diphosphate (10 mg. of histamine base) during the third, fourth, and fifth months of her pregnancy.

Only one event which might in any way be construed as representing an untoward effect on the pregnant uterus occurred in the entire series. A primigravida, 29 years of age, sustained one episode of slight vaginal bleeding twelve hours after a subcutaneous injection of 0.137 mg. of histamine diphosphate (0.05 mg. of histamine base). The month of the pregnancy in which this bleeding occurred is not known. This is one of the smallest amounts of histamine given to any patient in the group, so that the significance of this observation is questionable.

We wish to suggest on the basis of our experience that there is at least an apparent lack of a clinical oxytocic effect of histamine diphosphate when it is administered to pregnant women by the subcutaneous or intravenous route or by both routes in the therapeutic dosages mentioned. We offer no preferred explanation as to why the oxytocic effect described by so many authors and attested by abundant laboratory study did not occur. A few of the possible explanations which immediately present themselves are: (1) that the elevated histaminase level known to occur in pregnant women might be responsible for the inactivation of the injected histamine; (2) that the dosage of histamine used was not adequate to provoke a clinically observable oxytocic response; (3) that the altered neurogenic response in the twelve patients in this series who had multiple sclerosis may in some way in these patients have altered the predicted effect.

## 77 RETROBULBAR NEURITIS, TREATMENT WITH HISTAMINE

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WITH THE TECHNICAL ASSISTANCE OF EVELYN F. HELGERSON, M.A.

The typical syndrome of retrobulbar neuritis consists of lowered visual acuity, some form of scotoma in the visual field, and usually a normal appearing nerve head. In both the acute and chronic forms of this syndrome edema and interference of the blood supply to the optic nerve are evident. The axons of the optic nerve will withstand ischemia for considerable time before final degeneration begins. A vasodilating agent which will increase materially the blood supply to the optic nerve is the most satisfactory means of restoring its function. Spontaneous recovery may and frequently does occur, but in our experience unless it begins within three weeks after the onset of symptoms, it will not be complete.

Histamine is the most powerful vasodilating agent available for increasing the blood supply to the central nervous system; hence the rationale for its use in the treatment of retrobulbar neuritis. Compared with other forms of treatment it is more universally applicable, is less discommoding to the patient, does not necessitate hospitalization, and seems to result in more rapid and complete recovery of vision.

In this study we have employed a 1:250,000 dilution of histamine administered intravenously by the drip method at rates varying from 24 to 48 drops

per minute. The rate employed depended on the patient's tolerance. Treatment for one and one-half hours has been carried out daily or every other day for from one week to three and one-half years. No untoward reactions have been observed. One patient received 410 treatments. In the light of our recent experiences it is obvious that some of our earlier patients had inadequate treatment.

During the past six years we have administered histamine to sixty-one patients who had retinobulbar neuritis. Of these sixty-one patients, thirty-four were women and twenty-seven were men. The ages ranged from 9 to 49 years, with an average age of 30.5 years.

Eighteen patients previously had been given typhoid vaccine intravenously. None of the eighteen regained normal vision, seven obtained 25 to 75 per cent improvement in vision, ten noted no change, and one experienced greater loss of vision after treatment with typhoid vaccine. When these same eighteen patients were treated with histamine administered intravenously, five regained normal vision, five obtained 25 to 75 per cent improvement, and eight noted no change.

Forty-three of the sixty-one patients had not had previous treatment with typhoid vaccine but did receive histamine intravenously. Of these forty-three patients, nineteen regained normal vision, three obtained 75 per cent recovery, six, 50 per cent recovery, five less than 25 per cent recovery, and the remaining ten noted no change.

Twenty-two of the sixty-one patients had had visual loss for more than one year, five of these regained normal vision. Five of the sixty-one patients had had visual loss for six months to one year and none of these regained normal vision. Eight of the sixty-one had had visual loss for three to six months and six of these regained normal vision. Twenty-one of the sixty-one patients had had visual loss for one month or less, twelve of these regained normal vision. Five of the sixty-one patients were unable to furnish definite data as to the onset of their visual loss and of this group one obtained normal vision.

In summary, it is interesting to note that twenty-four of the sixty-one patients regained normal vision and that the vision of ten improved 50 to 75 per cent. Although eighteen of the sixty-one patients had had previous treatment with typhoid vaccine, none of the group had normal vision following this type of treatment. However, when histamine was administered intravenously to this same group of patients, five regained normal vision.

## 78 THE PREPARATION AND PROPERTIES OF MAMMALIAN STRIATED MYOFIBRILS

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A physicochemical method for isolation and purification of myofibrils from mammalian skeletal and cardiac muscle has been devised. Blocks of living fresh muscle were frozen and cut into thin sections with a freezing microtome. The sections were transferred directly to a proteolytic enzyme buffer solution ( $0^{\circ}\text{C}$ , pH, 7, ionic strength, 0.25). After digestion at  $0^{\circ}\text{C}$  for thirty to forty-five minutes, the myofibrils were separated by mild mechanical agitation. Following this, the segregated myofibrils were separated from other cellular and interstitial components of muscle by controlled centrifugation.

The isolated myofibrils which were obtained in large quantities by this method had characteristic physical properties. They varied in length and

breadth but retained the essential microscopic structural detail, plasticity and birefringence, characteristic of myofibrils in their normal intracellular location, prior to application of the procedure for isolation. They were soluble in a slightly alkaline reagent (0.5 normal potassium chloride plus 0.03 normal sodium bicarbonate) giving viscous solutions which displayed a strong birefringence of flow. In buffer solutions with an ionic strength of 0.15, the myofibrils dissolved on the acid side of pH 4.0 and on the alkaline side of pH 10.0. In phosphate buffer solutions with an ionic strength of 0.5 the myofibrils dissolved when solutions were on the alkaline side of pH 6.3. In phosphate buffer solutions with the ionic strength increased from 0.15 to 0.5 by addition of potassium chloride, the myofibrils were soluble when solutions were on the alkaline side of pH 6.0.

The isolated myofibrils, although probably modified in some respects by tryptic action and mechanical agitation during isolation at 0° C, exhibited the property of contractility when placed in dilute neutral solutions of adenosine triphosphate. Under these conditions myofibrils isolated from skeletal muscle of man and rabbit contracted rapidly so that long fibrillar structures with sharply defined microscopic characteristics were converted irreversibly into spherical masses with no recognizable structural detail.

## 79 CASTOR BEAN SENSITIVITY

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Five men working in a mill where castor bean is processed complained of allergic symptoms associated with this occupation.

The five were skin tested with seven extracts of castor bean products. All of them had a positive reaction to one or more of the extracts. Three of the five were then tested by the Prausnitz-Kustner passive transfer technique. The reagins identified by this method were identical with the positive tests obtained by direct skin testing. An extract of castor pomace gave a positive test in all five patients. All of the patients associated contact with this substance with their clinical symptoms.

Thirteen control subjects were skin tested with the same extracts. Eight failed to react to any of the castor extracts. Three gave slight irritative non-specific reactions. Two control subjects reacted in some degree to most of the extracts. The latter two were foundrymen suffering from asthma. The relationship of these positive tests to their asthma has not been determined.

## 80 BERYLLIOSIS: OBSERVATIONS AND REPORT OF CLINICAL STUDY OF SEVENTY CASES OF CHRONIC DISEASE

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Berylliosis may be defined as a general disease characterized chiefly by pulmonary insufficiency and having the major pathologic changes in the lung. It results from the inhalation of finely divided beryllium compounds.

The epidemiology and etiology of the disease are discussed, and a summary of the clinical study of seventy cases of chronic disease is presented. Report is made upon chemical findings in the tissues of persons with fatal cases and the pathologic anatomy of the disease is discussed briefly. Data on concentrations of beryllium in the air of plants are reviewed. X-ray films from representative cases will be presented.

## 81 CORRELATION OF LACTOBACILLUS COUNTS WITH EXTENT OF DENTAL CARIES IN AN INSTITUTIONAL POPULATION

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Lactobacillus counts from the saliva of more than 200 institutionalized children have been made in a State Laboratory by personnel especially trained to provide such service to the dentists of the state. Duplicate analyses of samples collected within twenty-four to forty-eight hours were made for most of the subjects. All samples were collected before breakfast and before cleaning of the teeth after the subjects chewed paraffin.

The individual lactobacillus counts were correlated with the extent of tooth decay (number of DMF surfaces), the rate of progression of tooth decay during the previous nine to twenty-one months, and the status of mouth hygiene. No correlation was evident except with the habitual and the current state of oral hygiene. The data offer nothing to support the premise that lactobacillus counts provide a diagnostic or prognostic index of the activity of dental caries. Any apparent relationship evidently relates to the hygiene of the oral cavity and the areas for lodgment of pabulum for bacterial growth rather than to the process of caries in itself.

## 82 ACUTE TORTICOLLIS DUE TO FOOD ALLERGY

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Repeated attacks of acute torticollis have been observed to follow the ingestion of allergenic foods in each of three specifically sensitized patients.

Although the symptoms of tightness, pulling, aching, and tenderness of the posterior cervical muscles, with and without associated headaches, have been observed as manifestations of chronic food allergy and such symptoms have been induced following the experimental ingestion of allergenic foods, instances of acute torticollis have not been described on this basis. As a rule, recurrences of cervical myalgia on an allergic basis do not go on to the development of acute torticollis.

The most favorable circumstances for the development of an allergic writhing neck include the presence of a high degree of specific sensitivity to a commonly ingested food which previously had been avoided for at least three or four days prior to an evening feeding of this food. This, in turn, is usually associated with an allergic reaction of such severity as to cause the patient to retire shortly after the evening meal. Motion on turning in bed during sleep, upon arising from bed the following morning, or within the first half hour after arising may be associated with the sudden onset of an excruciatingly severe pain sharply localized to a small exquisitely tender area in the trapezius or sternocleidomastoid muscles. Contracture of the involved muscles with immobilization of the head in a position favoring the shortening of the affected muscle or muscle groups immediately results as a protective measure because of the extreme pain on motion of the head. A relatively fixed position of the head and shoulders is maintained for one or two days following which the attack gradually subsides coincident with a decrease in the hypertonicity, aching, pain, and tenderness of the affected muscles.

### 83 THE EFFECT OF VARYING THE DOSE OF IODINE ON THE BEHAVIOR OF RADIOIODINE TRACERS IN PATIENTS WITH EXOPHTHALMIC GOITER

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WILLIAMS, PH D (BY INVITATION), AND M H POWER, PH D  
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Five patients who had exophthalmic goiter received repeated tracer doses of  $I^{131}$  by mouth, with varying quantities of stable sodium iodide as carrier. The behavior of each dose was followed by means of serial measurements of the intensity of radiation over the thyroid gland and abdomen, and of the concentration of  $I^{131}$  in blood serum and urine.

Where small quantities of iodine (1 to 100  $\mu$ g) were used a large proportion of the dose was accumulated and retained in the thyroid gland. The quantity of  $I^{131}$  in the thyroid gland increased exponentially for six to twenty-four hours and thereafter decreased very slowly.

When large quantities of iodide (10 mg) were employed little or none of the dose remained in the thyroid gland and a large proportion (80 per cent) appeared in the urine. Observations over the thyroid gland revealed a prompt but temporary accumulation in the thyroid gland which reached a peak representing 15 to 35 per cent of the dose within three hours. Subsequently, the radioiodine content of the thyroid gland decreased rapidly until at twenty-four hours only a small fraction remained. This decrease of  $I^{131}$  in the thyroid gland occurred at a rate comparable to but somewhat slower than the rate of disappearance of radioiodine from the blood.

When an intermediate quantity of iodide (10 mg) was used the curves describing the changes in concentration of  $I^{131}$  in the thyroid gland appeared to be composites of those obtained in the high and low carrier groups. The rate of disappearance of radioiodine from the blood and the quantities eventually accumulated in the thyroid gland or excreted in the urine likewise were intermediate between the other groups.

It is suggested that the iodine accumulating function of the hyperactive thyroid gland observed after the use of small doses of labeled iodide is largely or entirely related to the synthesis and storage in the thyroid gland of organic compounds containing iodine.

The function of the hyperactive thyroid gland which is observed when large doses of iodide are administered appears to be similar to that described by Astwood in the normal human thyroid gland blocked with antithyroid drugs and appears to represent the storage within the thyroid gland of iodide as such.

The function of the kidney with respect to the excretion of iodide did not appear to be materially altered over the range of doses herein employed.

### 84 EVALUATION OF THE USE OF ANTERIOR PITUITARY EXTRACT IN THE TREATMENT OF PITUITARY DWARFISM

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CYRIL M MACBRIDE, M D ST LOUIS, MO

This study was based on the treatment of eleven dwarfs with pituitary growth extract. Eight patients exhibited all of the characteristics of pituitary dwarfs. Case 4 had had satisfactory treatment for congenital syphilis. A twelfth dwarfed patient with normal epiphyses and genital development reached normal size in two years with treatment by desiccated thyroid 0.06 Gm daily.

alone Case 6 had had Pott's disease of the tenth dorsal vertebra Cases 3, 6, and 7, although having the appearance of pituitary dwarfs with genital and general physical underdevelopment, had no delay in epiphyseal development for their ages

*Method of Study*—After preliminary periods of observation with accurate anthropometric measurements, each of the eleven patients was given Phyone pituitary extract (Wilson), which contains both growth and thyrotropic factors. An initial dose of 0.25 c.c., then doses of 0.5 and 1.0 c.c. on alternate days were given intramuscularly. A 2.0 c.c. dose thrice weekly was continued for two to six months with control intervals without treatment.

Basal metabolic rates were normal in all but three patients. No patient had myxedema. Cases 1, 4, and 8 received daily doses of 0.03 to 0.12 Gm of desiccated thyroid daily in addition to Phyone treatment because of slightly subnormal basal metabolic rates. Case 11 received thyroid a short time to note any added effect on growth, no significant change was noted. The other eight patients receiving Phyone were not treated with any thyroid, androgen, or estrogens during their observation on Phyone treatment.

Blood counts, hemoglobin, urine analyses, and blood Kahn tests were normal. Roentgenograms of the skull were normal in nine patients, and two had bridging of the clinoids with small sella turcicae.

*Results*—Case 3 was the only one of the eleven whose height and weight at the end of treatment exceeded the minimum average height and weight for his age. The normal growth curves for boys and girls are shown in the same scale as the growth curves of this group for comparison.

Our patients failed to show an increase in stature or weight so definite that it could be attributed to the use of the growth extract Phyone alone.

*Conclusion*—Further studies with more potent pituitary growth hormones are needed before one can unreservedly recommend the use of commercial growth hormones for prolonged treatment of pituitary dwarfism.

## 85 EFFECT OF INSULIN ON TUBULAR REABSORPTION OF GLUCOSE IN DIABETIC PATIENTS

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In 1941 Shannon and associates reported a 10 per cent reduction of the renal tubular reabsorption of glucose ( $TM_G$ ) in four out of six dogs following an injection of 50 units of insulin. Creatinine was used to measure glomerular filtration. The ratios of  $TM_G$  subsequent to insulin divided by their values prior to insulin were 0.88, 0.89, 0.87, 1.06, 0.77, and 0.96.

In our studies five adult male patients, ages 18 to 33, known to be diabetic for one to eight years, were used. They showed no clinical or laboratory evidence of hypertension, renal disease, or other complications. They were readily desugared on insulin and were classified as insulin sensitive. Insulin dosages varied from 10 to 35 units of crystalline and from 20 to 50 units of protamine.

The patients were stabilized on diet and insulin. On the morning of the insulin experiment the daily doses of protamine and crystalline insulins were given, the glycemic level was raised, and the  $TM_G$  values were determined through three periods. Several days later after the protamine insulin had been withheld for not less than forty-eight hours and crystalline insulin for twenty-four hours, the experiment was repeated. The ratios of  $TM_G$  after the insulin

divided by their values without insulin were 0.98, 0.92, 0.83, 0.83 and 0.77 respectively for the five subjects. It has been generally recognized that variation from period to period in  $TM_G$  determinations may be large.

The plan used by other workers of averaging the values for three successive periods to secure the  $TM_G$  of the patient was followed. Although these values were depressed consistently after the administration of insulin, the degree of depression was not regarded as significant in view of the limitations of the method.

It should be realized that all of the patients had an endogenous supply of insulin in addition to the injected supply. One may therefore conclude only that a deficiency in insulin does not interfere significantly with tubular reabsorption of glucose.

## 86 EFFECT OF HYPERGLYCEMIA ON THE CLEARANCES OF INULIN AND PARA AMINOHIPPURIC ACID

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(INTRODUCED BY ROBERT W. KEFTON, M.D.)

Early in our studies of renal function in the presence of hyperglycemia certain discrepancies in the determination of PAH clearance were noted. Klopp, Young and Taylor reported that hyperglycemia may decrease the PAH clearance from 30 to 80 per cent but not effect the clearance of mannitol. It seemed wise to study this problem further.

In the present studies to be reported insulin was maintained at levels of 20 to 75 mg. per cent, the para aminohippuric acid (PAH) at 0.9 to 3.5 mg. per cent, and the glucose at normal fasting to hyperglycemic levels.

Thirteen patients of whom seven were women and six were men were studied. The previous clinical data had shown normal kidney function. The experiments were done in the following manner: (1) at normal glycemia and low levels of PAH, (2) at hyperglycemia and low levels of PAH, and (3) at hyperglycemia and high levels of PAH. The inulin clearance rose slightly but progressively under the experimental conditions enumerated. This rise was not significant since it fell within the limits of the standard deviation of the method. All values were corrected to a surface area of 1.73 square meters. At fasting blood sugar levels the PAH in women averaged  $593.8 \pm 126.3$  cc per minute, and at hyperglycemia  $407.1 \pm 146.1$  cc per minute. The average depression due to hyperglycemia was 31.4 per cent.

In the men the PAH<sub>c</sub> at fasting glucose levels averaged  $654.9 \pm 105.3$  cc per minute, and at hyperglycemia  $312.5 \pm 119.5$  cc per minute, with an average depression of 52.5 per cent. The depression of PAH<sub>c</sub> was reproduced on separate days in one patient. At fasting glucose levels the  $TM_{PAH}$  was 76.9 mg. per minute and at hyperglycemic levels the  $TM_{PAH}$  was 54.0 mg. per minute. The average depression due to hyperglycemia was 29.8 per cent.

Hyperglycemia depresses the PAH<sub>c</sub> and the  $TM_{PAH}$  values. Sufficient experiments have not been conducted to establish correction factors. The competitive antagonism between glucose and para aminohippuric acid as it affects tubular function is now under investigation.

## 87 CHOLESTEROL TOLERANCE TESTS IN NORMAL, DIABETIC, AND HYPERTENSIVE PATIENTS

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The possibility that intimal atherosclerosis is due in some instances to a basic metabolic fault in the capacity to utilize cholesterol caused us to devise a cholesterol tolerance test which will be briefly described

This test was performed on fourteen normal subjects, four patients with essential hypertension, eleven patients with diabetes mellitus, 23 to 79 years of age, one patient with hypercholesterolemia and xanthomatosis of the skin, one patient with nephrotic stage of chronic glomerulonephritis and hypercholesterolemia, one male patient, 25 years of age, with acute myocardial infarction and normal serum cholesterol

Repeated analyses established that the method entailed a maximal error of 5 per cent In the group of fourteen normal subjects, the total free cholesterol increased from 4 to 38 per cent, cholesterol ester increased 0.8 to 24 per cent Maximal increases were distributed from the second to the eighth hour In two subjects the free and ester forms fell 5 to 18 per cent

The results in the eleven diabetic patients were the same as in the normal subjects in all essential respects The results in four patients with essential hypertension likewise were within the range shown by normal subjects In the patient with skin xanthomas and in the patient with nephrosis, both of whom had hypercholesterolemia, the blood levels of both free and ester forms of cholesterol fell 3 to 19 per cent during the tolerance test

The only abnormal results were found in the 25-year-old man with acute myocardial infarction In his case the free cholesterol rose 43 per cent and the cholesterol esters rose 29 per cent The maximal rise in free cholesterol in the normal group was 38 per cent in one instance, with an average maximal rise of 17 per cent The maximal rise in cholesterol ester in the normal group was 24 per cent, with the average maximal rise of 9 per cent

These data do not demonstrate any difference between normal, hypertensive, and diabetic subjects in their response to a cholesterol tolerance test This indicates that the tendency of the diabetic and hypertensive patient to develop intimal atherosclerosis is not due to a prolonged or excessive postprandial hypercholesterolemia

## 88 THE MEDICAL AND METABOLIC FACTORS IN THE SURGICAL MANAGEMENT OF HYPERTHYROIDISM

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The goitrogens are by no means solely responsible for the decreased morbidity and mortality following the modern thyroidectomy There are a multitude of factors which must still be considered in the proper surgical treatment of the patient with hyperthyroidism in addition to the use of the goitrogens

A summary of consecutive thyroidectomies on 400 goitrous patients from 1937 to 1942, and prior to use of our present regimen, revealed 185 to be for nontoxic goiter and there resulted no hospital deaths Two hundred fifteen were for hyperthyroidism and these accounted for an unusually high degree of morbidity and mortality so that the total mortality averaged 2.25 per cent During this same period four pole ligations were done and ten thyroidectomies were staged, followed by a high mortality The causes of death included thyroid crisis, respiratory failure, massive atelectasis, bronchopneumonia, acute cardiac failure or shock, and liver death These represent to a great extent avoidable



causes of death in the light of our present methods of treatment. Consequently, a summary of consecutive thyroidectomies on 400 goitrous patients treated by our present regimen from 1943 to 1948 revealed 157 to be for nontoxic goiter, and there occurred no deaths. Two hundred forty three were for hyperthyroidism which is a greater number than in the former group. Furthermore, the severity of the hyperthyroidism in this group was usually greater and the complications were more frequent. Yet there resulted no deaths. No ligations were done and only five thyroidectomies were staged but with no deaths.

In our experience, there are two groups of medical and metabolic factors which have led to this lowered morbidity and mortality rate of the modern operation. The first includes seven general factors and major developments which have made all modern surgery less hazardous. The second group embraces at least nine specific factors related to hyperthyroidism which have counteracted the deleterious hypermetabolic effects of the disease.

The clinical use of the goitrogens and internal radiotherapy is still in many ways in the experimental stage. When a physician contemplates the use of one of these substances he should seriously consider the general and specific factors in the treatment of toxic goiter in addition to the new substance in order to serve the best interests of the patient.

## 89 THE BETA GLUCURONIDASE ACTIVITY OF HUMAN ENDOMETRIUM

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Endometrial biopsies (suction curette) were obtained from thirty two women at various days of the normal menstrual cycle. The specimens were weighed, homogenized, and assayed for  $\beta$  glucuronidase activity using the method of Fishman, Springer and Brunetti (J Biol Chem 173:449-456, 1948).

The  $\beta$  glucuronidase activity increased during the first two thirds of the menstrual cycle, followed by a decline particularly in the last three days of the cycle. The presence or absence of progesterational endometrium seemed to bear little reference to these changes. These results would indicate that the  $\beta$  glucuronidase activity of endometrium parallels the estrogen secretion reported for the menstrual cycle.

Multiple specimens of endometrium were obtained from the same patients and assayed independently. While there was some variation in results, it was insufficient to disturb the general pattern described here.

$\beta$  glucuronidase is believed to play a fundamental role in the physiologic action of the estrogenic hormones. The cyclic change in activity observed in the endometrium parallels the histologic effect of estrogen stimulation. Therefore it is possible that endometrium normally participates in the metabolic conjugation of estrogenic hormones.

## 90 DIFFUSE PANCREATIC CALCIFICATION WITH DIABETES MELLITUS

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(INTRODUCED BY MAURICE HARDGROVE, M.D.)

The present study is one of the association of pancreatic calcification with diabetes mellitus. In addition to four cases observed clinically, there are three cases of pancreatic calcification from which autopsy findings are presented. It

has been suggested that disseminated pancreatic calcification be recognized as a distinct clinical entity. We feel that it is impossible to differentiate clinically disseminated calcification from multiple calculi in the pancreas. The etiology of the change is unknown. Alcoholism was the one most frequent, suspicious, predisposing cause in our cases. The symptoms simulated those of gall bladder colic, perforated ulcer, and appendicitis. It is suggested that a scout film be taken in acute abdominal conditions generally and in a diabetic patient particularly. In this way more cases will be recognized and erroneous surgery will be prevented. Also, with the advent of recent advances in pancreatic surgery, it is conceivable that partial or total pancreatectomy may be helpful in relieving the intractable pain present in some of the patients.

## 91 PSEUDOHYPOPARATHYROIDISM

### CASE REPORT

M. G. PETERMAN, M.D., AND J. L. GARVEY, M.D. (BY INVITATION)  
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A 12-year-old girl was examined because of logorrhea, mental retardation, and convulsions. The patient's condition had been diagnosed as epilepsy and hypothyroidism. The mother was ill during pregnancy and almost miscarried in the fourth month. Delivery and infancy were normal. The infant was always hungry, never lost infantile fatness, always had a hoarse voice, and often a crowing inspiration. At 2 years she complained of numbness and tingling in the extremities.

The patient weighed 101 pounds and her height was 58½ inches. She had a round, pudgy face with a slightly yellow pallor of the skin and a generalized myxedematous appearance. Chvostek's sign was strongly positive as were the ulnar and peroneal reflexes. The Trousseau test was negative. The voice was hoarse and coarse. There were several areas of calcification in the skin. The mouth was kept in a "carp" position.

The girl had repeated episodes of sudden squealing or shouting with pupils dilated and without loss of equilibrium. The mouth was held open, the extremities were tense. There were five to fifteen seizures in each twenty-four hours.

The serum calcium was 6.6, phosphorus, 11 milligrams. The urinary phosphorus excretion was 0.7 Gm. in twenty-four hours. The Sulkowitch calcium test of the urine was negative throughout.

Lateral x-ray of the skull showed scattered areas of calcification in the basal nuclei and generalized osteoporosis. This osteoporosis was also found in all of the long bones.

The electrocardiogram showed a marked increase in the Q-T interval.

$$K = \frac{Q-T}{RR} = 51$$

The electroencephalogram showed spiking, slow 6 per second waves, a big build-up on hyperventilation with 3 per second waves not typical of the dysrhythmia of epilepsy.

The Binet-Simon test gave an I.Q. of 79, the Rorschach test "there is much in the structure of the record and in the child's behavior during the test to indicate an organic brain disturbance of an epileptic or convulsive type."

An intravenous pyelogram showed normal kidneys and ureters. The Mosenthal test was negative.

There has been a good response to treatment with high calcium, low phosphorus acid ash diet. Treatment with parathyroid extract was ineffective. Treatment with acid ash high calcium, low phosphorus diet plus Hytakerol brought the blood chemistry to normal. However, the seizures have persisted.

No case of hypoparathyroidism or of the pseudo type has been reported with osteoporosis of the bones.

## 92 PREMATURE CALCIFICATION OF THE COSTAL CARTILAGES ITS FREQUENT ASSOCIATION WITH PSYCHONEUROSES AND POSSIBLE ENDOCRINE IMBALANCE

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(INTRODUCED BY S. B. GRANT, M.D.)

Calcification of the costal cartilages is a process which, while presumed to be a matter of ageing, is frequently found in young persons.

It is generally considered that this deposit is a matter of no consequence. For this reason, perhaps very little progress has been made in determining the factors which cause or are associated with the calcification. Association with various organic diseases, such as pulmonary tuberculosis has been postulated but disproved.

A study of 277 consecutive patients under the age of 40 years has been made. One hundred fifty eight of these were shown by x-ray to have costal cartilage calcification, while 119 did not. In addition to age and sex the following details from the case history were noted: the complaints, the state of nutrition, the basal metabolic rate, the blood calcium, history of menstrual disorders in the women and the final chief diagnosis.

Significant differences between those persons who showed calcium in the costal cartilages and those who did not were found in the number of chief complaints, the history of menstrual disorders, the percentage with obesity and the final chief diagnosis. In the former group there were twice the number of complaints, twice the incidence of obesity and three times the incidence of menstrual disorders. Most remarkable was the fact that the complaints were *not* due to organic disease five times as often in the calcification group.

It is suggested that premature calcification in the costal cartilages may be evidence of a link between psychiatric and previously unrecognized endocrine disturbances. Further experimental work on young persons showing this phenomenon is needed.

## 93 PSYCHOSOMATIC PROBLEMS IN A PRIVATE PRACTICE

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This analysis was made from the office records of two internists in private practice in a community of 15,000 which has a large agricultural farming area. Heavy industry with its attendant physical and psychological problems is not a factor.

To determine the incidence of functional problems 300 consecutive complete office studies were analyzed. Each patient in the study had been subjected to detailed history, complete physical examination and a minimum laboratory and roentgen survey consisting of urine analysis, blood count, sedimenta-

tion rate, and chest fluoroscopy, with chest film if considered necessary. Additional appropriate laboratory or other procedures had been utilized where indicated.

It is fully realized that an analysis of this type cannot be entirely objective, and that the personal equation must enter into the classification of certain patients. As nearly as possible, those patients with demonstrable organic disease which might explain the symptoms were eliminated from the functional or psychosomatic classification. Essential hypertension, peptic ulcer, and urticaria were classified as organic disease in this survey.

The 300 cases comprised 119 men and 181 women.

Of the 300 cases, ninety-two, or 31 per cent, were considered to fall within the functional or psychosomatic classification. These ninety-two cases comprised thirty men and sixty-two women. On the basis of these figures, 25 per cent of all the men studied and 34 per cent of all the women studied could be so classified. Men constituted 40 per cent of the total series and 33 per cent of the functional problems.

An attempt was made to classify the primary complaint of each of these ninety-two patients within an organ system.

SYSTEM	CASES
Circulatory	27
Respiratory	3
Gastrointestinal	21
Urinary	2
Neuromuscular	6
Endocrine	1
Visual	1
Oral (buccal lesions)	1
General (fatigue, tension, headache)	30

A separation of the three major classifications according to sex is as follows:

SYSTEM	TOTAL	MEN	WOMEN
Circulatory	27	12	15
Gastrointestinal	21	5	16
General (fatigue, etc.)	30	9	21

The oldest woman in the functional series was 69 years of age, the youngest, 13. Fifty-five per cent were under 40 years of age which would appear to minimize the climacteric factor in production of symptoms. The oldest man was 67 and the youngest 21.

Analysis of causative factors was difficult. Anxiety, in one guise or another, appeared to be of major importance.

Beneficial results of management coincided closely with the time, patience, and practical common sense devoted to each case.

## 94 THE EFFECT OF ENTEROGASTRONE CONCENTRATES ON GASTRIC SECRETION IN HUMAN BEINGS

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We have studied the effect of enterogastrone concentrate on the gastric secretory response of human beings to the injection of histamine and to the ingestion of a test meal. An enterogastrone concentrate prepared for parenteral administration and one prepared for oral administration were used in this study. The activity of the material prepared for parenteral administration was tested on rats and dogs before it was used in human volunteers.

The enterogastrone preparation given parenterally produced a threefold to fourfold reduction in the volume of gastric juice secreted by rats after pyloric ligation. When the enterogastrone was given intravenously in doses of 100 mg or more to dogs with Heidenhain pouches secreting in response to histamine, pronounced inhibition of acid output uniformly occurred.

Enterogastrone concentrate given intramuscularly to ten human volunteers in doses of 200 mg did not affect significantly the gastric secretory response to the injection of histamine during a double histamine test. Enterogastrone concentrate given to fourteen human volunteers in doses as large as 400 mg intramuscularly and 18 Gm orally did not significantly affect the gastric secretory response to a modified Ewald test meal.

Although the extract of hog's intestinal mucosa used for parenteral administration inhibited gastric secretion in rats and dogs, the immediate conclusion that this was due to enterogastrone was tempered by the fact that toxic reactions sometimes accompanied its use in dogs. Because contaminating substances may have prevented action by enterogastrone in human beings, we do not feel that our results can at this time be interpreted to indicate that pure enterogastrone will be without action in similar tests on human beings.

#### 95 A COMPARISON OF THE TWELVE HOUR NOCTURNAL GASTRIC SECRETION IN UNCOMPLICATED DUODENAL ULCER BEFORE AND AFTER HEALING

ERWIN LEVIN, M.D. (BY INVITATION) JOSEPH B. KIRSNER, M.D. AND  
WALTER LINCOLN PALMER, M.D., CHICAGO, ILL.

The twelve hour nocturnal gastric secretion was measured in thirteen patients with duodenal ulcer during a period when the ulcer was easily demonstrable roentgenologically and when typical distress was present. The studies were repeated in the same individuals after medical treatment had led to healing of the ulcer roentgenologically and to the complete subsidence of symptoms.

During the period of active ulcer the volume for the entire group averaged 1047 cc, the free acidity of the total volume averaged 58 clinical units, and the average output of acid 2208 milligrams. After healing of the ulcer, there was no significant change in the average nocturnal gastric secretion, the volume averaging 1,002 cc, the free acidity 54 clinical units and the output of acid 1957 milligrams. A significant decrease after healing was noted in the volume in one patient, in the concentration of acid in two and in the output of acid in one individual. An increase in gastric secretion after healing was observed in a similar number of cases.

The average nocturnal gastric secretion in patients with healed duodenal ulcer is significantly greater than that found in a group of thirty three healthy normal individuals studied under identical conditions. The secretion of acid in all the patients was continuous and was maintained at a relatively higher level than is seen in normal individuals. In no instance were there periods of anacidity for as long as one hour such as are frequently encountered in normal persons.

The persistence of hypersecretion in the vast majority of cases after healing of duodenal ulcer emphasizes the importance of continued careful antacid therapy in such patients.

## 96 THE INCIDENCE OF SYMPTOMS AND THE GASTRIC SECRETORY RESPONSE TO HISTAMINE IN PATIENTS WITH AND WITHOUT CHRONIC GASTRITIS

JOHN W FINDLEY, JR, M D (BY INVITATION), JOSEPH B KIRSNER, M D, AND  
WALTER LINCOLN PALMER, M D, CHICAGO, ILL

To evaluate more completely the clinical significance of chronic gastritis in patients with gastrointestinal symptoms, a comparative analysis was made of the symptomatology and of the gastric secretory response to histamine among four groups of cases (1) fifty patients with atrophy of the gastric mucosa, (2) fifty with superficial gastritis, (3) fifty with hypertrophic gastritis, and (4) 100 individuals in whom the gastric mucosa appeared normal gastroscopically. The absence of other organic disease was established by normal physical examinations, blood counts, urinalyses, normal proctoscopies, and by normal x-rays of the gastrointestinal tract.

The symptoms, with few exceptions, did not vary significantly among the four groups. The incidence of epigastric pain, the most frequent complaint, ranged from 44 per cent among patients with atrophy to 72 per cent among the normal subjects. The type, location, and pattern of pain were not distinctive for any of the four groups. There were, likewise, no significant differences in the incidence of nausea, vomiting, anorexia, constipation, weight loss, weakness, bad taste, or sore tongue. Diarrhea was described by 22 per cent of patients with atrophy as compared with 4 to 12 per cent for the other groups. The incidence of hematemesis in hypertrophic gastritis was 6 per cent, superficial gastritis, 2 per cent, and none in the remaining groups. A history of melena was encountered in 4 to 6 per cent of patients with gastritis and in one of the normal patients. Numbness or tingling of the extremities was reported by 6 per cent of patients with atrophy of the gastric mucosa and in 0 to 2 per cent of the remaining subjects. The duration of symptoms was essentially the same in all four groups.

Histamine achlorhydria was present in 51 per cent of forty-seven patients with atrophic gastritis, 27 per cent of forty-one with superficial gastritis, 9 per cent of forty-five with hypertrophic gastritis, and 3 per cent of ninety-five normal persons. A quantitative analysis of histamine tests performed in 165 patients indicated, as might be expected, that the smallest amounts of acid were secreted by patients with atrophy of the gastric mucosa, with superficial gastritis next. The output of acid in patients with hypertrophic gastritis, contrary to previous assumptions, did not differ significantly from that of normals.

The present data indicate that chronic gastritis is not an important cause of gastrointestinal symptoms.

## 97 BLEEDING PEPTIC ULCER, A REPORT OF ONE HUNDRED SIXTY CASES

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EVANSTON, ILL

In 1938 a report was made on the eighty patients with bleeding peptic ulcer admitted to Evanston Hospital in the decade 1928 to 1937. The present report is an analysis of the 160 patients with bleeding peptic ulcer admitted in the succeeding decade, 1938 to 1947. It is made to evaluate further trends in management and results thereof.

The results of the present analysis indicate that the starvation (or strict Sippy) regimen is falling into disuse in a general hospital practice. The early use of a relatively liberal and balanced diet has found favor with most attending physicians. The results of this policy are shown by the further reduced time of bleeding, reduced amount of bleeding, lessened emergency surgery, shortened hospital stay, and lowered mortality.

In the present series there is no instance of surgery to control bleeding, and only three had elective surgery, which was done after the hemorrhage and anemia were under control.

It is of interest to note that ascorbic acid blood levels were determined in fifty seven cases of varying clinical background. A few had levels within normal range, but the majority who had distinctly lowered levels of ascorbic acid in the blood showed the most prolonged and severe hemorrhage.

The increased availability of blood in recent years has led to the treatment of some of these patients with repeated transfusions (up to 6,000 cc). In this group there is no conclusive evidence of increased bleeding. On the other hand, there is no conclusive evidence that repeated transfusions are of value in shortening or reducing severity of hemorrhage although they are the outstanding means of therapy for shock.

## 98 PYLORIC BALANCE IN ILEUS, CONCEPT AND APPLICATION

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Ileus is a common clinical picture occurring as a complication of a variety of disorders, and the nature of the primary etiologic agent may become only a matter of speculation unless the patient is seen early in the episode. Mechanical small intestinal obstruction may supervene in peritonitis or vice versa, the factor of depletion of circulating protein by shifts into the peritoneal cavity and the edematous bowel wall occurs in each condition and further impairs intestinal tonus.

In the evaluation of the patient who is seen after several days of illness or in following the development of postoperative complications, any additional method of obtaining precise information is valuable. The application of continuous suction to the indwelling nasal catheter is of therapeutic merit but if such decompression is used blindly and without consideration and measurement of the components involved, valuable information is not obtained, time is lost and actual harm (dehydration and demineralization of the patient) may be done. When properly employed it affords a unique method of evaluating the degree of impairment of gastrointestinal function and yields data of great diagnostic and prognostic significance.

Quantitative studies begun by the author in 1930 led to the formulation of the term pyloric balance to express the volume and direction of flow of gastrointestinal secretions through the pylorus per day. In ileus prognostic value lies in the fact that the quantity of the negative balance (lost to the body economy) varies characteristically with the nature of the crippling agent. Volumes greater than 1500 cc are seen in mechanical small intestinal obstruction, rarely exceed 1 liter in peritonitis and are less than 500 cc in the adynamic ileus that follows mere handling of the bowel at operation, particularly in patients with lowered circulating protein. With this factual knowledge the crippling mechanism may be identified more certainly and appropriate treatment selected.

If a mechanical factor is clearly present, direct attack on the obstructing agent (band or volvulus) should be made if the general condition of the pa-

tient is good, particularly if the obstruction is jejunal. Enterostomy without exploration remains a most useful method of decompression of obstructed ileal loops, especially in the depleted patient or in those who are intolerant of prolonged nasal intubation. Distal emptying will occur after a few days as spasm and edema of the bowel wall at the site of obstruction subside and as hunking, plastic exudate is absorbed. Nasal catheter suction remains the treatment of choice for ileus in which no extrinsic mechanical factor is present, watch being kept for evidence of an unresolved abscess or infected hematoma which may require drainage.

## 99 DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS OF ACIDITY VARIATIONS IN THE STIMULATED AND NONSTIMULATED STOMACH

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Alvarez and others have pointed out that the determination of the acidity in one gastric aspiration does not give reliable information concerning the gastric acidity status. Similarly, variations in the gastric acidity have been noted during fractional gastric analyses. In the present study we were impressed by marked variations in the acidity in the stimulated and unstimulated stomach not only from day to day but also during each fifteen-minute period and during the time of the experiment, and by their diagnostic and therapeutic implications.

Thirty-five patients with ulcer and four normal controls were tested for free and total acidity at fifteen-minute intervals for one- to two-hour periods daily for five to ten days under standard conditions. Gastric stimulation was produced by 0.1 c.c. of histamine diphosphate 1:1,000 solution per 10 kilograms of body weight.

Gastric acidity in nonstimulated ulcer patients varied from 25 to 143 units on successive days during the same period of time (8 to 10 A.M.). In the controls it varied from 0 to 19 units. Following histamine injections the acidity response varied from 6 to 96 units in the patients with ulcer and from 26 to 57 units in the controls. In one patient with gastric ulcer tested at almost weekly intervals, the acidity ranged from 0 to 29 units before and 11 to 62 units following histamine stimulation during a period of three months.

Gastric acidity varies at any given time over a period of days or weeks whether stimulated by histamine or not. These well-known variations may be caused by one of many influences acting upon an individual throughout the day, and should be taken into consideration when the therapeutic effect of any substance is evaluated, inasmuch as otherwise erroneous conclusions can be made by either condemning or approving a certain substance, depending upon the gastric secretory activity on that particular day.

Only prolonged observations on a large number of patients can give a clearer picture concerning the effect of a substance on gastric secretion. Although the daily variations are marked (as seen from the tables), the average obtained from a large sample (over 200 analyses) results in an almost flat curve.

In evaluating substances for ulcer therapy by testing them for their antacid effect, the periodic variations in gastric acidity occurring normally must be carefully considered.



# 100 GASTRIC ACIDITY RESPONSE DURING THE INTRAVENOUS ADMINISTRATION OF PROTEIN HYDROLYSATES

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The gastric secretagogue effects of protein and products of protein digestion when given orally are well known but the effects of intravenously administered protein hydrolysates on gastric acid secretion have been studied by few workers. During an investigation of the clinical uses of protein hydrolysates such observations on the gastric secretory response were made in a moderately large number of patients who received various types of protein hydrolysates with water or glucose as diluents.

After a control period of gastric secretion obtained during a saline infusion for forty five to sixty minutes one of the protein hydrolysates was infused at about 72 drops per minute and the gastric aspirations continued for another hour at fifteen minute intervals. Six types of protein hydrolysates (varying according to their preparation and diluent) were studied. Each proteolysate was tested a minimum of eleven and a maximum of twenty four times. 162 tests being performed altogether.

All solutions stimulated gastric acidity to varying degrees and in varying percentages of the patients tested. Complete vagotomy (five patients) and thoracic sympathectomy and splanchnicectomy (three patients) did not change the foregoing observations. Plasma infusion caused no rise while 10 per cent dextrose solutions caused a decrease in the gastric acidity.

The increase in gastric acidity during the intravenous infusion of protein hydrolysates may be due to various factors which require further investigation. The same proteolysates in a dextrose solution caused a smaller rise in gastric acidity and in a smaller number of patients. The pH of the solution seemed to be a factor also. Thus the hydrolysates with a pH of 4 gave a higher acidity response than those with a pH of 6.5 to 7.0 (81 to 31 per cent respectively in forty three tests). While the histamine content of these mixtures could be considered the cause of the gastric acid response following their intravenous administration, the results did not bear this out clearly. The hypoglycemia suggested by some observers as a cause for increased gastric secretion was not encountered in our patients.

These observations suggest that various known and some unknown factors may cause increased gastric acidity during protein hydrolysate infusions. In the clinical use of protein hydrolysates therefore one must attempt to eliminate at least the known various factors which possibly increase the gastric acidity, because such an increase may be harmful in some patients.

# 101 EXPERIENCES WITH NEEDLE BIOPSY OF THE LIVER IN HEPATIC CIRRHOSIS

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Needle biopsy of the liver has revealed the presence of hepatic cirrhosis in sixty-six patients seen at the Cincinnati General Hospital during the past four and one half years. The cirrhosis was classified as nutritional (alcoholic) in thirty seven, postnecrotic in seventeen, biliary in four and was unclassified in eight. In the course of the clinical study, the following laboratory tests were

performed in most of the cases cephalin-cholesterol flocculation, thymol turbidity, biomsulfalein excretion (5 mg per kilogram of dye, retention in forty-five minutes), and serum bilirubin concentration. In over one-third of the cases total serum protein and albumin/globulin ratios were determined. The clinical diagnosis of cirrhosis was confirmed by the biopsy in fifty-five patients. In the remaining eleven, cirrhosis was first recognized as the result of the liver biopsy.

An attempt was made to correlate the histopathologic changes with the results of the various tests. The morphologic alterations were classified according to the degree of fatty vacuolization, cellular degeneration and necrosis, bile stasis, inflammatory reaction, and fibrosis. In the group of thirty-seven patients with nutritional (alcoholic) cirrhosis such a correlation study has been accomplished. Jaundice or hyperbilirubinemia was present in twenty of these patients, the thymol turbidity was increased in fifteen and normal in twenty, the cephalin-cholesterol flocculation was positive in thirty and negative in seven, biomsulfalein retention was present in twenty-nine and absent in five. Except for more marked fibrosis in the patients with either positive cephalin flocculation tests or abnormal biomsulfalein retention, there was no direct correlation between the results of the tests and the character of the histopathologic changes noted.

## 102 THE BLOOD AND BONE MARROW IN PATIENTS WITH CIRRHOSIS OF THE LIVER

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DETROIT, MICH

The peripheral blood and bone marrow findings in cirrhosis of the liver have been analyzed on the basis of a review of the literature and the authors' study of twenty-five patients with diagnoses verified by biopsy of the liver. The principal blood findings are macrocytic or normocytic anemia with normal or elevated mean corpuscular hemoglobin values, lymphopenia, and thrombocytopenia in the majority of the cases. Anemia may be independent of bleeding, and the severity of the anemia or macrocytosis does not appear to be related to the severity or duration of the liver lesion, although this appears to be true of experimental cirrhosis in rats. Absolute lymphopenia, regardless of the total leucocyte count, is the most constant significant alteration of the leucocyte picture.

The consistent change in the bone marrow is extension of the marrow organ so that active hematopoiesis is found in the shafts of the long bones. Regardless of the presence or absence of bleeding or anemia, the marrow of the sternum is of normal or increased cellularity, with normal or increased erythroidogenesis and megakaryocytopoiesis in most cases.

Even in patients with advanced liver lesions hypocellularity of the marrow is an unusual finding, in spite of peripheral anemia which is often characterized by a lack of signs of accelerated regeneration of red cells.

Macronormoblastic erythropoiesis is seen in patients with macrocytic anemia, but megaloblastic erythropoiesis does not result from hepatic cirrhosis.

The presence of peripheral cytopenias (anemia and thrombocytopenia) in spite of normal or increased formation of erythroblasts and megakaryocytes in the marrow is suggestive of hypersplenism. The well-known involvement of the spleen in patients with hepatic cirrhosis is additional evidence in favor of this view.

In patients with chronic hemorrhage, the blood and sternal marrow pictures are those of iron deficiency anemia although other changes such as lymphopenia and thrombocytopenia tend to persist

The changes described in the blood and bone marrow are not considered pathognomonic of hepatic cirrhosis even though they appear to be characteristic of the disease. The combined blood and sternal marrow study is useful in establishing the diagnosis of cirrhosis of the liver in patients in whom other diseases have obscured its manifestations or in whom historical evidence was absent so that the clinical diagnosis was difficult to make

### 103 THE ROUTINE USE OF THE SERUM FLOCCULATION REACTION WITH HAYEM'S SOLUTION

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(INTRODUCED BY HANS POPPER, M.D.)

A serum flocculation test with Hayem's\* solution has been described by Gros as being a fine indicator of hepatic disease. The simplicity of his method appeared to justify an attempt at using it as a screening test. A modification of the original Gros test necessitated by apparent shortcomings was employed routinely in a series of 500 patients. The technique consists chiefly in mixing equal volumes of serum and Hayem's solution. Development of a precipitate within twenty-four hours is considered a positive result.

Seventy-two patients had a positive H (Hayem's) test. They may be classified as follows: acute hepatitis five, cirrhosis of liver ten, prolonged obstructive jaundice (two to six months) three, neuro and cardiovascular syphilis, one, syphilitic chancre two, active infectious or rheumatoid arthritis eight, acute rheumatic fever one, severe congestive failure two, chronic lung infections (including pleuritis) fourteen, acute infectious diseases, fourteen, neoplastic diseases, eight, pyonephrosis one, thyrotoxicosis two, and scleroderma, one.

In all cases of this group and in many of the H negative group, one or more of the following tests were carried out: sedimentation rate, cephalin flocculation, thymol turbidity, zinc sulfate turbidity, and Takata-Ara. The incidence of abnormal results of these tests was considerably higher in the H positive than in the H negative group. Likewise, hyperglobulinemia was far more frequent in H positive than in H negative sera. A significant increase in gamma globulin as determined by the Cohn-Wolfson method was found in all of the thirty-five H positive sera in which this determination was performed.

In some instances a positive H reaction was the only pathognomonic sign at the time of the patient's first examination, followed sooner or later by the discovery of the responsible disease (cirrhosis, metastatic neoplasm, pyonephrosis, pleuritis, active tuberculosis). No false positive reaction was encountered. All of these seventy-two patients were seriously ill, even though the initial symptoms did not always betray this fact.

The H negative group of 428 patients included a variety of conditions as they are encountered in the average hospital population. Among them were ten cases of acute hepatitis and three of (recent) obstructive jaundice, but none of cirrhosis of the liver.

The conclusion is warranted that the H test is capable of revealing diseases causing serum protein alterations. Hyperglobulinemia and particularly, increases of gamma globulin appear to be chiefly responsible for a positive reaction.

Mercury bichloride 0.5 sodium sulfate 50 sodium chloride 100 distilled water 100.00

Consequently it may reveal not only a hepatic disorder but also systemic infections and malignant diseases. A negative test does not rule out any such conditions except perhaps cirrhosis of the liver. These findings and the simplicity of the test would favor its adoption as a routine clinical procedure.

#### 104 THE INFLUENCE OF INGESTION OF VARIOUS LIPIDS UPON THE THYMOL TURBIDITY

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The thymol turbidity depends on the interplay of various factors among which the serum lipids appear the one most easily influenced experimentally. Hence the thymol turbidity was determined in 163 patients with various diseases before and three and six hours after the intake of various lipids. The turbidity rose, in general, after the ingestion of almost every type of lipid, the highest rise being encountered after administration of 50 Gm. of butter. The addition of 6 Gm. of choline to the butter usually enhanced the rise in the thymol turbidity. Choline alone elevated the thymol turbidity significantly less than butter with choline or butter alone. In five patients in whom the elevation of the thymol turbidity after the administration of butter and choline was compared with the rise of the serum phospholipids and total lipids, the slope of the thymol turbidity curve paralleled far more that of the serum phospholipids than that of the total lipids. The same was found in three patients in whom the response of the thymol turbidity, serum phospholipids, and total lipids was determined after the intake of either butter or choline alone. It was therefore concluded that the response of the thymol turbidity to the intake of choline or butter reflects primarily an elevation of the serum phospholipids. This is in keeping with the reported high concentration of phospholipids in the serum precipitate produced by the addition of thymol and by the known relation of choline to the serum phospholipids. The zinc sulfate turbidity—reflecting primarily gamma globulin (Kunkel)—was determined in almost all instances simultaneously with the thymol turbidity, it represents an important supplement to the diagnostic use of the thymol turbidity and is to a much lesser degree influenced by lipid intake.

The alimentary rise of the thymol turbidity was on the average over 3 units in control subjects. It was depressed to a statistically significant degree (to about 1 unit) in infections, carcinoma, cirrhosis, and obstructive jaundice, and also in cardiac and wasting diseases. The smallest response, if any, was noted in gastrointestinal diseases. In acute infectious hepatitis the postprandial rise was almost normal. The response of thymol turbidity to the intake of butter and choline can thus be considered as a simple clinical test of intestinal absorption of lipids, most probably reflecting the phospholipids.

#### 105 COMBINATION OF FLOCCULATION TESTS IN THE DIFFERENTIAL DIAGNOSIS OF JAUNDICE

FREDERICK STEIGMANN, M.D., HANS POPPER, M.D., AND BERNARD H. SHULMAN, M.D. (BY INVITATION), CHICAGO, ILL.

In 455 patients, 262 with jaundice, the cephalin cholesterol flocculation, thymol turbidity, and thymol flocculation were performed, and in almost all of them also the Takata-Ara, colloidal red, Gros (flocculation with Havem's solution), and zinc sulfate turbidity (Kunkel) tests.

Most sensitive in the recognition of liver damage in order of decreasing frequency, were the Gros test, the zinc sulfate turbidity, thymol turbidity, colloidal red test, cephalin cholesterol flocculation, thymol flocculation, and Takata Ara test.

In general, all flocculation tests were abnormal in a much higher percentage of patients with medical (patent extrahepatic biliary tract) than with surgical (obstructed extrahepatic biliary tract) jaundice. However, the number of false positive or negative tests with each individual test was too high to permit much reliance on the results of individual tests in the differential diagnosis. Purulent hepatitis produced by bacterial infection of the portal triads shows, though surgical in nature, the same results in the flocculation tests as the medical types of hepatitis. However, liver cell damage caused by biliary obstruction alone (biliary hepatitis) reveals as a rule, negative or only slightly positive flocculation tests. If the possibility of a purulent hepatitis which can be recognized clinically by signs of septicemia is considered, the diagnostic value of the individual flocculation tests is somewhat increased. If the results of cephalin flocculation, thymol turbidity, Gros test and zinc sulfate turbidity are dovetailed with each other by bringing them into a system which considers the degree of alteration of these tests, the percentage of those wrongly diagnosed as medical (false positives) was reduced to 35 per cent and of those wrongly diagnosed as surgical (false negatives) to 13 per cent of all jaundice cases.

In the differentiation of cirrhosis from infectious or toxic hepatitis, zinc sulfate turbidity and Gros tests are of particular value; they are highly pathologic in cirrhosis even with only slightly increased cephalin flocculation and thymol turbidity and moderately pathologic in acute hepatitis even if cephalin flocculation and especially thymol turbidity are greatly elevated. The Takata Ara test is positive in cases with highly elevated zinc sulfate turbidity.

A quantitative correlation of the results of several flocculation tests may be useful for the differentiation between medical and surgical jaundice on the one hand and acute and chronic hepatitis (cirrhosis) on the other.

## 106 INFLUENCE OF ENDOGENOUS AND NUTRITIONAL FACTORS UPON THE PLASMA VITAMIN A ALCOHOL

HANS POPPER, MD, FREDRICK STEIGMANN, MD, HATTIE DANIEWICZ, PHC  
(By Invitation), and ALVIN DUBIN, MS (By Invitation), CHICAGO, ILL.

The total plasma vitamin A level reflects only vaguely temporary changes in vitamin A nutrition (it is reduced only in prolonged deficiency) and is significantly influenced by endogenous factors for example liver diseases or infections. Vitamin A in plasma and tissues occurs in either the free (alcohol) or the esterified form. Four fifths of the total plasma vitamin A is normally in free form, whereas the liver contains primarily esterified vitamin A.

The question arises as to the influence of nutritional as well as endogenous factors upon the fractions of the total plasma vitamin A. In 276 control subjects and patients suffering from various diseases the vitamin A esters only rarely were decreased below the normal (around 10  $\mu\text{g}$  per 100 cc) but were significantly increased in nephrosis and erratically increased in various illnesses, especially liver diseases. The alcohol fraction, however, was significantly reduced (from about 40 to almost 15  $\mu\text{g}$  per cent per 100 cc) in carcinoma, malnutrition, wasting diseases, infections (such as pneumonia) and liver diseases especially cirrhosis, in which the lowest levels (for example 2  $\mu\text{g}$  per 100 cc) were observed. Under pathologic circumstances the esters may represent even under fasting conditions more than 90 per cent of the total plasma vitamin

A Endogenous hypovitaminemia A, therefore, is caused almost entirely by a reduction of the alcohol fraction, the esters often even being elevated. This effect can be explained by impaired release of vitamin A alcohol from the damaged liver due to inactivation of vitamin A esterase which normally converts the liver vitamin A ester to alcohol. Presence of esterase could be demonstrated in human liver and serum.

Intake of 75,000 units of vitamin A in aqueous or oily menstruum raises the plasma vitamin A esters for several hours. They may then represent in controls 80 per cent of the total vitamin A since the alcohol level does not rise during this period (twelve cases). Prolonged effect on vitamin A nutrition, however, was reflected in the plasma vitamin A alcohol. In eighteen persons who were given alternately for one to two weeks a diet containing less than 100 units of vitamin A daily or a normal diet with daily supplements of 10,000 units vitamin A, the plasma vitamin A alcohol level was, as a rule, significantly higher during the supplemented period than during the vitamin A poor period. A few exceptions found under pathologic conditions require further explanation. The vitamin A esters did not reflect the vitamin A intake, often being elevated during the vitamin A poor period.

It is concluded that both endogenous and general nutritional factors influence primarily the vitamin A alcohol level and that the latter, being independent of postprandial effects, represents a more sensitive index of vitamin A nutrition than the total vitamin A level.

# 107 STUDIES OF THE INFLUENCE OF OXYQUINOLINE DRUGS ON GROWTH OF ENDAMOEBA HISTOLYTICA AS MEASURED IN BLOOD IODINE LEVELS OF MAN

ALVA A. KNIGHT, M.D., AND JEANNE MILLER, M.A., CHICAGO, ILL.

(INTRODUCED BY ROBERT W. KEETON, M.D.)

In the study of iodine absorption in the oxyquinoline drugs in man, we found blood levels of iodine to be approximately 100 gammas in Anayodin and chiniofon, 400 gammas in Vioform, and 800 gammas in Diodoquin.

We then began the study of growth curves of *Endamoeba histolytica* in mixed bacterial flora in media consisting of equal parts of human serum and egg yolk infusion. Counts of both the amoeba and the bacteria in the inoculum were made, and these counts were repeated in the inoculated media at twelve, twenty-four, forty-eight, seventy-two, and ninety-six hours. The peak growth of *E. histolytica* occurred at forty-eight to seventy-two hours, while the bacteria continued to grow profusely for a longer period without diminution.

The patient was then given one of the oxyquinoline drugs for seven to ten days and the blood again drawn and its iodine content in gammas determined. Culture media of equal parts of serum and egg yolk infusion was again prepared and the inoculations made as before. The counts of *E. histolytica* and bacteria were made at the same time intervals as before. A total of over forty patients was studied.

Inoculated media containing 100 gammas of iodine showed a slow growth of *E. histolytica* continuing to seventy-two and ninety-six hours, but not the continuing rise to forty-eight or seventy-two hours previously shown. Growths became less active as the iodine level rose, and between 250 and 300 gammas no growth beyond forty-eight hours was shown. Levels of 400 to 600 gammas showed a progressively shorter growth period—rarely was any growth present at twenty-four hours.

The bacterial growth was uninfluenced by the varying iodine levels.

Studies in fecal concentrations of iodine have been made on a few patients. Other iodides are being studied in like manner and are to be reported on at a later date.

## 108 THE CLINICAL EVALUATION OF DUODENAL SUBSTANCE IN CHRONIC ULCERATIVE COLITIS

MICHAEL H. STRFICHER, M.D., CHICAGO, ILL.

Duodenal substance was administered to thirty-five patients who had chronic ulcerative colitis. The dosage used varied from 12 to 48 tablets daily, each  $\frac{1}{4}$  Gm. administered uninterruptedly for one year. No other specific medication was used. The patients were on a controlled diet.

Comparative clinical evaluations were made as to the frequency of exacerbations in the disease before and after intake of duodenal substance, frequency of stools, presence of blood in the stools, gain or loss in weight, exaggeration or alleviation in abdominal cramping, and the general well-being of the patients under treatment.

The results obtained in 85 per cent of the patients were very favorable.

1. Exacerbation of the disease was noted in three instances.
2. The majority of the patients gained weight.
3. The majority of the patients felt better and ate better while receiving duodenal substance.
4. Duodenal substance may be considered a very valuable aid in the therapy of chronic ulcerative colitis.

## 109 THE CYTOLOGIC DIAGNOSIS OF CANCER OF THE STOMACH: PRELIMINARY REPORT

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(BY INVITATION), ALEX. B. RAGINS, M.D. (BY INVITATION), AND  
JACOB MEYER, M.D., CHICAGO, ILL.

The need for additional diagnostic methods for the purpose of detecting gastric neoplasms is well known. While the tested techniques of x-ray and gastroscopy are adequate in most cases, the results of these examinations not infrequently are equivocal or negative in the presence of a malignant tumor of the stomach.

The recent application of the cytologic technique in the diagnosis of cancer of the uterus has given impetus to wider application of the method in the diagnosis of neoplasms of the respiratory and genitourinary tracts. This method has been further utilized to a limited degree in the detection of carcinoma of the stomach.

In undertaking this study it was our purpose to evaluate its effectiveness both as an individual test and as a test complementary to roentgenography and gastroscopy in a large general hospital.

The method employed has been that of fasting gastric lavage with physiologic salt solution. The sediment obtained is smeared on glass slides, the smears being relatively thick. The stain which we have found most reliable is LA 36 (Papaneolaou). In addition we have also used the method of formalin fixation and paraffin section, staining the latter with stain LA 36 and with the standard hematoxylin and eosin stains.

All of the patients studied had clinical histories suggesting the possibility of gastric neoplasms. In the beginning we employed thin smears in forty-nine

instances, but because of the paucity of cells, this technique was abandoned. This report is based upon the study of 175 cases employing the thick smear technique. The paraffin section method has been used concomitantly in approximately 150 of these cases. Diagnoses in these cases included (1) carcinoma of the stomach, (2) gastric ulcer, (3) duodenal ulcer, (4) benign gastric neoplasms, and (5) gastritis and a variety of extragastric diseases.

Of the 175 cases, about thirty-five cases of gastric carcinoma are included. We have been able to make definitely positive diagnosis in approximately 35 per cent of these cases. Those cases of carcinoma of the stomach in which we were unable to make a positive cytologic diagnosis were cases in which the smears, because of the presence of debris and poorly preserved cells, allowed no interpretation. The two most important factors which prevent our making a positive diagnosis are (1) gastric retention and (2) the presence of large, necrotic tumors. The other factor is the infiltrating type of tumor without mucosal involvement. The proportion of false positive reports should not exceed 10 per cent, and we feel that this figure will be reduced even further. The results of the paraffin section studies parallel those of the smear study, the hematoxylin and eosin stains seem to be preferable when the former technique is used. In several instances we failed to find tumor cells preoperatively, and washings of the resected specimens also failed to reveal malignant cells.

This is a preliminary report. The series is as yet too small and the method too new to give statistically valid results. In the individual case, however, when other methods of examination give equivocal or negative results, a positive cytologic diagnosis may solve the problem.

## 110 COMPARATIVE NITROGEN BALANCE STUDIES OF ORAL PROTEIN DERIVATIVES AND NATURAL PROTEIN IN PROTEIN-DEFICIENT PATIENTS

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Abundant evidence exists proving that oral protein digests are both practical and biologically efficacious. A question which logically arises is, if patients are capable of ingesting a processed protein, what advantages does such a preparation offer over natural protein? A metabolic study in which synthetic and natural proteins are evaluated in the same protein-depleted patient has not to our knowledge, been presented.

The opportunity to conduct such a study on three severely hypoproteinemic and hypoalbuminemic subjects presented itself in the course of a metabolic project designed to determine the relative biologic indices of a preparation of gluten with and without 1 per cent lysine. After having been on a regimen of protein therapy calculated to produce bare nitrogen equilibrium for periods of from thirty-four to fifty-five days, the patients showed no signs of clinical improvement or of regeneration of serum proteins or albumins. The patients were then given gluten with 1 per cent lysine\* to the point of tolerance for periods of from eight to ten days, then equivalent amounts of natural proteins such as meat, eggs, and milk, and finally they were allowed diets combining maximal quantities of both. Daily nitrogen analyses of urine, stool, and ingested protein were carried out. Body weight, total serum protein, and serum albumin determinations were made at the beginning and conclusion of each period.

\*Provided by Interchemical Corporation



*Results*—The maximal quantities of gluten with 1 per cent lysine ingestible averaged 22.8, 26, and 28.6 Gm nitrogen per day for patients weighing 44.3, 52.0 and 55 kilograms respectively. This produced positive nitrogen balances of 9.3, 9.4, and 10.9 Gm nitrogen per day respectively.

The maximal quantities of natural protein ingested ran 24.8, 26.5 and 28.6 Gm nitrogen per day in the same order as previously given. The positive nitrogen balances were 7.5, 11.8, and 9.7 Gm nitrogen per day in the order described.

The patients were able to ingest mixtures of the two sources of protein in the average ratio of two parts of the protein derivative to one part natural protein. Accordingly 51.0, 49.7 and 55.8 Gm nitrogen per day, respectively were ingested (on the basis of body weight this was 6 Gm or more protein per kilogram per day). This produced positive nitrogen balances of 18.8, 18.4 and 30.6 Gm nitrogen per day respectively.

Total serum protein concentration increases for all three periods were 1.5, 3.3, and 2.2 Gm per 100 cc respectively. Increases in serum albumin concentration were 1.1, 2.1 and 2.0 Gm per 100 cc respectively.

Gains in body weight of 6.1, 7.0 and 6.3 kilograms, respectively, were noted at the end of the experiment.

#### *Conclusions*—

(1) In two out of three protein depleted patients gluten with 1 per cent lysine produced a significantly better nitrogen balance than did similar quantities of natural protein. In a third patient the reverse was true.

(2) Combining a protein derivative with natural protein enabled the patients to ingest considerably more nitrogen than possible with either one alone.

(3) The ingestion of 6 or more grams of protein per kilogram per day was therapeutically feasible.

(4) Significant responses in total serum proteins, serum albumin and body weight were seen.

### 111 EXPERIENCES WITH A MERCURIAL DIURETIC FOR SUBCUTANEOUS INJECTION

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A new mercurial diuretic prepared for subcutaneous administration has been tried clinically in office and hospital practice. Thiomerin is an organo-mercury compound combined with mercapto acetate forming a mercaptide. It is a readily soluble white powder which is diluted with water to contain 40 mg of mercury per cubic centimeter. Preliminary reports show it to have a low incidence of local or systemic toxicity in animals.

The present report includes observations on thirty patients who have received from 1 to 50 subcutaneous injections of Thiomerin. The patients had all been under our observation and most of them required frequent intravenous injections of one of the mercurial diuretics for control of congestive failure. Patients were maintained on ammonium chloride in dosage of 4 to 6 Gm daily preceding and during the course of the present observation. Digitalis was continued if the patient was previously digitalized. Changes in the morning weight and also clinical observations of edema and pulmonary congestion were used as criteria of diuretic effect. The dosage was usually 1 cc, but ranged from 0.25 to 2 cc. Following injection, diuresis began in two to six hours and lasted from twelve to forty eight hours. The weight loss was proportional to the

amount of edema and was usually two to five pounds, with extremes of zero to fifteen pounds. Three patients, followed for three months, have maintained a steady edema-free weight by substitution of Thimerin for intravenous mercurials.

The injections have been surprisingly free from local reaction. One patient developed a sore nodule, still present after one month, another complained of local tenderness, lasting one week. About one-third of the patients experienced systemic symptoms, ranging from slight muscle cramps to severe prostration, cramps, and diarrhea, lasting from twelve to seventy-two hours. The more severe reactions were associated with greater urine output, although there was considerable individual variation. Usually subsequent injections, even with comparable diuresis, were attended by lesser or absent reactions. In four patients the use of Thimerin was discontinued because of the severity of the symptoms. We have found Thimerin a satisfactory diuretic for subcutaneous administration in the majority of patients. There is a low incidence of local reactions. Systemic reactions are more frequent than are seen after intravenous mercurials. When these occur, they are related to the degree of diuresis and can usually be prevented by the use of smaller doses.

## 112 RENAL EXCRETION OF SALICYLATE DURING TREATMENT OF ACUTE RHEUMATIC FEVER

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CHICAGO, ILL.

Clearance studies were carried out in twelve patients who were convalescing from acute rheumatic fever but were still taking large doses of aspirin (6 to 9 Gm. daily). Urine salicylate was partitioned into total salicylate, free salicylate, and salicylurate by the method of P. K. Smith and colleagues. Chloride and creatinine clearances were likewise determined. After these tests the subjects were placed on an alkalinizing regimen with doses of 2 to 33 Gm. of sodium bicarbonate every six hours for three days in addition to the regular aspirin medication. The clearance tests were then repeated. The urinary pH was noted in both sets of experiments. Plasma salicylate was found to be practically entirely unconjugated and was calculated as if it were all unbound, even though from 60 to 90 per cent has been found by other investigators to be bound to protein. To rule out the effect of alkali on the glomerular filtration rate, the apparent free salicylate clearances were expressed as ratios of salicylate to creatinine clearance ( $SA/C \times 100$ ), as were chloride clearances ( $Cl/C \times 100$ ).

During the initial tests, when the urine pH ranged from 4.98 to 5.99,  $SA/C \times 100$  ranged from 0.84 to 2.43, while  $Cl/C \times 100$  ranged from 0.45 to 3.16. In other words, the free salicylate ions which were filtered by the glomeruli were reabsorbed by the tubules almost as completely as chloride, if the urine was strongly acid. In the second set of experiments, when the urinary pH ranged from 6.22 to 7.98,  $SA/C \times 100$  ranged from 4.6 to 22.8 or from two to eight times as high as when the urine was acid. The true clearance of unbound plasma salicylate would have been three to five times higher than these values. In other words, in the presence of excess sodium ions, free salicylate was cleared almost as well as mannitol. This phenomenon would account for the lowering of plasma salicylate levels when all alkali is given.

The ratio of free salicylate to total salicylate in the urine was found to be proportional to the pH of the urine, being as low as 0.10 when the urinary pH

was about 50 and as high as 0.66 when the pH was 7.75. Thus alkalinization did not affect the excretion of conjugated salicylates which were apparently already maximally high. In fact, with the absence of detectable amounts of conjugated salicylates in the plasma, it appeared that conjugated salicylates were either formed in the renal tubules or secreted by them like diatrizol or hippurates.

Chloride creatinine clearance ratios were not found consistently increased after alkalinization. Since the excretion of chloride is affected by many factors which could not be controlled in these experiments these results are not surprising. Yet the dependence of both chloride and salicylate excretion on the amount of fixed base available for excretion would make it appear that both compete with each other for the available base and that under the conditions of acid urines both are for the most part returned. This might explain the high plasma chloride and relatively low sodium concentrations found during salicylate therapy.

### 113 THE RECORDING OF OXYGEN SATURATION OF HEMOGLOBIN BY A WHOLE BLOOD OXIMETER AND ITS APPLICATION TO CLINICAL INVESTIGATION

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ROCHESTER, MINN.

(INTRODUCED BY H. B. BURCHELL, M.D.)

A photoelectric oximeter has been devised by means of which the degree of oxygen saturation of whole blood can be measured instantaneously and continuously, independent of the total content of hemoglobin with a degree of accuracy sufficient to warrant its use in place of the Van Slyke gasometric analysis procedure in a variety of clinical and research applications.

The method is based primarily on two physical principles: (1) that of measurement of concentration of the chief blood pigments, oxyhemoglobin and reduced hemoglobin, in terms of their transmission of light according to Beer's law and (2) the differential light absorption characteristics of these pigments in two spectral regions.

Whole blood, either in the form of a small sample or in continuous flow is contained in a polythene tube which has an internal volume of 0.5 cc. and is transilluminated by a constant intensity light source. The emergent light is recorded in two selenium barrier layer photoelectric cell circuits, one filtered to pass the red light wave lengths (650 to 750 millimicrons) which are transmitted almost exclusively by oxyhemoglobin, the other filtered to record in the near infrared (750 to 1000 millimicrons) where both oxyhemoglobin and reduced hemoglobin transmit with approximately the same facility. Since the red cell current represents blood concentration of oxyhemoglobin and the infrared cell current that of total hemoglobin, a ratio of red to infrared currents constitutes a measure of the relative concentration of the two pigments and therefore of the relative degree of oxygen saturation of the hemoglobin. By empirical calibration of the apparatus against results obtained by the Van Slyke method, absolute values, in terms of percentage of oxygen saturation, can be read directly from a nomogram or from a single calibrated galvanometer scale. The method also lends itself to photographic recording of saturation values synchronously and continuously with recordings of other physiologic variables.

To date this instrument has been utilized chiefly for studies of oxygen saturation of arterial blood and, in diagnostic cardiac catheterization where it allows immediate correlation of roentgenographic, intracardiac pressure and oxygen saturation observations

Accuracy of this photoelectric method has been computed from the results of simultaneous photoelectric and Van Slyke determinations carried out on 131 blood samples ranging in hemoglobin concentration from 13.0 to 25.8 Gm per 100 cc. The standard deviation of the differences between values obtained by the two methods was 1.8 indicated saturation per cent. This implies a 95 per cent likelihood that any saturation determination made by the whole blood oximeter will be within 3.6 per cent saturation of that obtainable by Van Slyke analysis.

#### 114 THE DETERMINATION OF HEMOGLOBIN AS PYRIDINE HEMOCHROMOGEN

A. HUGHES BRYAN, M.D., T. FRANKLIN WILLIAMS, M.A. (BY INVITATION),  
AND CELIA R. WEBB, A.B. (BY INVITATION), CHAPEL HILL, N.C.

For use in nutrition surveys a hemoglobin method was sought which would be accurate, reproducible, and convenient to use in field studies, allowing the blood to be analyzed in a central laboratory some hours after collection in the field. Of the many published hemoglobin methods, we selected the pyridine hemochromogen method of Rimington, modified it, and adapted it to the Beckman spectrophotometer and the conditions imposed by field studies.

*Method*—Ten cubic millimeters of blood measured in a calibrated Sahli type pipette are discharged into 1 cc of distilled water in a tube calibrated to contain 5 cc. The resulting hemolyzed blood is allowed to stand at room temperature twenty-four hours before analysis.

The pyridine hydrosulfite reagent is prepared fresh for each batch of analyses as follows. Five grams of sodium hydrosulfite powder is dissolved in approximately 80 cc of N/10 sodium hydroxide. The solution is filtered, 5 cc of pyridine are added, and the combined reagent diluted to 100 cc with N/10 sodium hydroxide.

To the hemolyzed blood are added 0.5 cc of pyridine hydrosulfite reagent and 1 cc of N/5 sodium hydroxide, and the reaction mixture is diluted to 5 cc with N/10 sodium hydroxide. The tube is shaken after each addition and foaming can be dispelled by the use of one drop of n-butyl alcohol. The density is determined in the Beckman spectrophotometer after ten to sixty minutes at 558 to 560  $m\mu$ . Pyridine hemochromogen has a very sharp absorption peak in the neighborhood of 558 to 562  $m\mu$ . For accurate work it is necessary to determine the point of maximum absorption for each set of determinations. The hemoglobin content of the blood is obtained from a calibration curve prepared by submitting to the foregoing procedure bloods whose hemoglobin content was determined by analysis for iron. A straight line relationship exists between hemoglobin content and density of pyridine hemochromogen.

We have investigated the influence of the following variables: (1) proportions of sodium hydrosulfite and pyridine in the reaction mixture; (2) effect of the order of adding the reagents; (3) change of absorption with time after adding the reagents; and (4) effect of the time of standing of the hemolyzed blood before addition of the reagents. The conditions described appear to give the most satisfactory and reproducible results. Preliminary work indicates that the error in repeated single determinations on the same blood is less than  $\pm 3$  per cent.

## 115 THE CELLULAR COMPOSITION OF THE BONE MARROW IN NORMAL INFANTS AND CHILDREN

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Extensive reviews of the literature on the cellular composition of the bone marrow in children have shown the lack of information available on this subject and have expressed the need for further investigation.

This study covers 138 sterl marrow punctures on normal individuals from birth to 19 years of age. There was very little if any variation by age found in the cell counts of individuals ranging in age from 1 to 19 years. The findings of the cell counts of this age group were therefore used to establish normal average values for the cellular distribution of the bone marrow (myeloblasts, 1.23 per cent, leucoblasts, 1.44 per cent, promyelocytes, 1.8 per cent, myelocytes, 3.46 per cent\*, metamyelocytes, 36.05 per cent, polymorphonuclear cells, 12.9 per cent, eosinophiles, 3.58 per cent, pronormoblasts, 0.47 per cent, basophilic normoblasts, 1.69 per cent, polychromatic normoblasts, 18.2 per cent, orthochromatic normoblasts, 2.72 per cent).

Cell counts on infants less than 1 year and particularly less than 1 month old showed wide and constant variations with age. The number of nucleated red cells during the first and second days of life approximated those established as normal averages (23.12 per cent). From then up to the fifteenth day there was a marked decrease, followed in turn by an increase to average values. Lower figures were again recorded during the third to fifth months. Among the individual cells of this series the polychromatic normoblasts showed variations identical to those of the total red cell group. Pronormoblasts and basophilic and orthochromatic normoblasts showed similar findings but the fluctuations in number became less marked and statistically less significant with the smaller number of cells. Over the entire span of nineteen years there seemed to be a tendency for the younger cells to decrease in number with progressing age and for the older cells to increase during the same period. This variation in percentage was minimal yet it occurred with remarkable consistency.

The observations on the myeloid series likewise brought strong and constant changes during the first month of life with less significant variations during later months and years. The values for the first and second days of life closely approached the average figures (60.59 per cent); the following days we saw a marked increase in number of myeloid cells with a drop around the twentieth day, followed by a rise to average figures by the end of the first month. From then on there was little deviation from the average up to the end of the observation period (nineteen years). As to the individual cells in this group the metamyelocytes which form the largest part of the series showed the same pattern as the total group. The smaller groups of the myeloid series showed findings similar to those of the total group but again the variations were not as marked because these cells occurred in much smaller numbers. In considering the total period of observation there seemed to be again a tendency for the number of younger forms to decrease and for the number of older forms to increase.

The myeloid erythroid ratio showed during the first and second days figures close to the established average (2.94) followed by a sharp rise up to the end of the second week (14.5), when the values again fell reaching the average at the

All cells showing more than kidney shaped indentation of their nucleus were placed among the metamyelocytes thus producing relatively low values for the myelocyte.

To date this instrument has been utilized chiefly for studies of oxygen saturation of arterial blood and, in diagnostic cardiac catheterization where it allows immediate correlation of roentgenographic, intracardiac pressure and oxygen saturation observations

Accuracy of this photoelectric method has been computed from the results of simultaneous photoelectric and Van Slyke determinations carried out on 131 blood samples ranging in hemoglobin concentration from 13.0 to 25.8 Gm per 100 cc. The standard deviation of the differences between values obtained by the two methods was 1.8 indicated saturation per cent, this implies a 95 per cent likelihood that any saturation determination made by the whole blood oximeter will be within 3.6 per cent saturation of that obtainable by Van Slyke analysis

#### 114 THE DETERMINATION OF HEMOGLOBIN AS PYRIDINE HEMOCHROMOGEN

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*Method*—Ten cubic millimeters of blood measured in a calibrated Sahli type pipette are discharged into 1 cc of distilled water in a tube calibrated to contain 5 cc. The resulting hemolyzed blood is allowed to stand at room temperature twenty-four hours before analysis.

The pyridine hydrosulfite reagent is prepared fresh for each batch of analyses as follows. Five grams of sodium hydrosulfite powder is dissolved in approximately 80 cc of N/10 sodium hydroxide. The solution is filtered, 5 cc of pyridine are added, and the combined reagent diluted to 100 cc with N/10 sodium hydroxide.

To the hemolyzed blood are added 0.5 cc of pyridine hydrosulfite reagent and 1 cc of N/5 sodium hydroxide, and the reaction mixture is diluted to 5 cc with N/10 sodium hydroxide. The tube is shaken after each addition and foaming can be dispelled by the use of one drop of n-butyl alcohol. The density is determined in the Beckman spectrophotometer after ten to sixty minutes at 558 to 560  $m\mu$ . Pyridine hemochromogen has a very sharp absorption peak in the neighborhood of 558 to 562  $m\mu$ . For accurate work it is necessary to determine the point of maximum absorption for each set of determinations. The hemoglobin content of the blood is obtained from a calibration curve prepared by submitting to the foregoing procedure bloods whose hemoglobin content was determined by analysis for iron. A straight line relationship exists between hemoglobin content and density of pyridine hemochromogen.

We have investigated the influence of the following variables: (1) proportions of sodium hydrosulfite and pyridine in the reaction mixture, (2) effect of the order of adding the reagents, (3) change of absorption with time after adding the reagents, and (4) effect of the time of standing of the hemolyzed blood before addition of the reagents. The conditions described appear to give the most satisfactory and reproducible results. Preliminary work indicates that the error in repeated single determinations on the same blood is less than  $\pm 3$  per cent.

karyocytes is observed in some cases of Hodgkin's disease. These cells are not specific for this reason. These "lymphoid" megakaryocytes may produce atypical forms of platelets. (3) Reed Sternberg cells were not seen in sternal aspiration material nor in the histologic sections of bone marrow particles from such material. Evidence is presented to show that the giant cells of Hodgkin's granuloma are not similar nor related to bone marrow megakaryocytes. (4) The finding of increased numbers of normal or atypical plasma cells, eosinophiles and reticular cells is neither a constant nor a specific pattern of the bone marrow in Hodgkin's disease. Eosinophilia of the bone marrow cannot be correlated with the peripheral blood eosinophilia. (5) The accurate diagnosis of the disease is established only by the finding of the specific pleomorphic lesion of Hodgkin's granuloma. This is best observed in the histologic section of an involved lymph node. The finding of any one cell or group of cells in sternal aspirated bone marrow films is insufficient evidence for the diagnosis of Hodgkin's disease.

#### 117 ANEMIA OF PREGNANCY TREATED WITH MOLYBDENUM IRON COMPLEX

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Four and six tenths per cent of our pregnant patients have a hemoglobin concentration less than 10 grams per 100 ml. but if the nonpregnant standard of 12 grams is used 39 per cent are anemic. We believe that the lower limit of the hemoglobin concentration between the twelfth and thirty-sixth weeks is 10 grams and from then to term is 10.5 grams. Up to twelve weeks antepartum and by six weeks post partum the lower limit is the normal nonpregnant limit or 12 grams of hemoglobin. All patients are screened by a hematoerit determination every three months at term and again at six weeks and six months post partum. Any patients with hematoerits less than 37 for the nonpregnant or early pregnant or less than 30 between the twelfth and thirty-sixth weeks or less than 32 between thirty-six weeks and term have a hemoglobin determination, an erythrocyte count and the various indices calculated.

Treatment with ferrous and ferric iron alone or with various vitamin combinations did not cause a significant increase in the rate of hemoglobin formation. A molybdenum iron complex has resulted in a rapid increase in hemoglobin concentration. If this does not occur within three weeks then more extensive studies should be made to determine the cause of the anemia.

#### 118 OBSERVATIONS ON THE TREATMENT OF ACUTE LEUCEMIAS WITH ANALOGUES OF FOLIC ACID

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(INTRODUCED BY RAYMOND GREGORY, M.D.)

Ten patients belonging to the group with acute leukemia have been treated with Aminopterin (4-amino-pteroylglutamic acid) or Methopterin (10-methyl-pteroylglutamic acid). Acute lymphocytic leukemia (leucosarcoma) was the diagnosis in seven instances and acute monoblastic leukemia in three. Eight patients were children and two were adults.

In general the patients were treated with 2 mg of the compound, administered intramuscularly, on alternate days. Some patients, receiving Methopterin, had the dosages increased to 2 mg daily. Blood transfusions and antibiotics were administered when indicated.

*Patients Treated With Aminopterin*—Three patients received Aminopterin only, a fourth patient received Aminopterin following an initial period on Methopterin. All four developed evidences of toxic reactions as manifested by stomatitis, macular rash, and marked depression of bone marrow activity. One of this group showed some clinical and hematologic improvement. One patient has died, and the remaining two have failed to show a definite response.

*Patients Treated With Methopterin*—Methopterin was given to six patients and a seventh received Methopterin but later was treated with Aminopterin. Of these seven patients, two died before the drug had a chance to act. Of the remainder, none developed manifestations of toxic symptoms in contrast to the patients treated with Aminopterin. One patient has developed a very conspicuous clinical and hematologic remission with a reversal of the marrow to a nearly normal picture. Another patient improved while receiving Methopterin but her condition deteriorated when subsequently receiving Aminopterin. Three patients have so far shown no improvement but are still under treatment.

The impression has been gained that Methopterin is superior to Aminopterin in the treatment of acute leucemias, especially in view of its lower toxicity.

## 119 TREATMENT OF ACUTE LEUCEMIA WITH AMINOPTERIN (4-AMINO-PTEROYLGLUTAMIC ACID)

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Remissions characterized by clinical and hematologic improvement and a return of the marrow pattern to normal in acute leucemias treated with Aminopterin were reported by Farber and co-workers, in the spring of 1948. The duration of treatment in this preliminary report was of three months or less. This report deals with our experience in the treatment of eleven patients with acute leucemia since May 1, 1948.

The action of Aminopterin is not clearly understood. Farber observed certain pteroylglutamic conjugate, notably Thiopurin and Diopurin, induced an accelerating effect on leucemic tissue. The search for an antagonist drug led to the synthesis of Aminopterin, this product caused a rapid fall in the total blood leucocytes, a reduction in the size of the hematopoietic organs, and aplasia of the marrow.

In our series there were nine children under 10 years of age, one adolescent 13 years of age, and one adult, 66 years of age. All but one had acute untreated leucemia. The duration of the disease prior to treatment was less than one month in ten patients. The drug was administered intramuscularly. The daily dose was adjusted to the age and weight of the patient and varied from 25 to 1 mg in the children and from 1 to 2 mg in adults. Daily administration was continued until clinical and hematologic effects were obtained, thereafter treatment was given four to five times a week until toxic manifestations developed. Transfusions were used to support the anemia. Hematologic effects were followed by serial blood and bone marrow studies. The duration of treatment has extended more than four months in four cases, two months in three, one month in two, and less than one week in two. Of eleven patients treated, eight



are still living, three are dead. Of the eight living patients, five sustained a remission three did not. Of the three patients who died one was treated in the terminal phase and one succumbed to a cerebral hemorrhage one was the adult. In the five patients in whom complete remissions were obtained, the duration of the clinical and hematologic improvement lasted for more than six weeks in two instances, after which some degree of enlargement of the hematopoietic organ and/or return to the leucemic marrow pattern recurred. Both of these patients are still living. In three cases, the duration of the remission is less than a month. Of the three living patients who showed no remission two were treated continuously four months and one for three weeks.

The remissions were initiated in each instance by a fall in the total number of blasts in the peripheral blood, and a rapid reduction in the size of the hematopoietic organs. The effects occurred within ten to twenty days after instituting treatment. Hyperpyrexia, abdominal tenderness and severe hemorrhagic diatheses were often encountered. In all instances a severe aplasia of the marrow accompanied these signs. The total dose of Aminopterin administered before signs of marrow aplasia developed varied from 2.5 to 20 mg. The drug was discontinued when aplasia developed. Following the aplastic stage the total leucocyte count rose gradually followed by a rise in the reticulocytes, erythrocytes and platelet levels. Granulopoiesis returned in the bone marrow within five to ten days. During the period of active marrow granulopoiesis, clinical improvement was dramatic and evidenced by a drop in the temperature curve and general appearance of good health. Treatment was resumed as soon as granulopoiesis was reestablished. Stomatitis developed in seven patients under prolonged treatment. In the two under treatment for four months who sustained a complete remission relapse of the symptoms occurred after six weeks and the clinical course has been subacute. Relapse was initiated by increase in size of the peripheral lymph nodes and spleen, a rising white count and recurrence of the leucemic marrow pattern. Under continued treatment reduction in the hematopoietic organs was obtained again. However the bone marrow has remained leucemic. In spite of the marrow involvement the red count hemoglobin and platelet values remained normal since the initial remission, a period now of more than three months. In the three patients under continuous therapy for four months the clinical course has been subacute and organ involvement has been minimal but no measurable improvement in the marrow pattern has been noted on repeated examinations.

**Summary**—Of eleven patients with acute leucemia treated since May 1, 1948, remissions have been obtained in five instances. The remissions have been characterized by a severe marrow aplasia followed by a rapid regeneration. Remissions are not sustained. Whether the use of this product will significantly change the course of the disease remains to be proved.

## 120 TREATMENT OF CHRONIC LEUCEMIA WITH A FOLIC ACID ANTAGONIST

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Previous reports on the use of pteroylglutamic acid antagonists in man have dealt primarily with the treatment of acute leucemia. This study is based on observations of the effect of the folic acid antagonist 4-amino pteroylglutamic acid\* in nine patients with chronic leucemia. One of the patients having my

\*-4-[(4-aminino-6-pteridinyl)-methyl]-amino-benzoyl]-glutamic acid

elogenous leucemia received two series of treatments. The drug was taken orally, in daily doses ranging from 1.25 to 7.5 mg over periods from six to twenty-five days. Results are shown in Tables I and II.

TABLE I LEUCOCYTE COUNTS IN PATIENTS WITH MYELOGENOUS LEUCEMIA BEFORE AND AFTER TREATMENT

CASE	TOTAL W B C PER C MM	STEM CELLS PER C MM	IMMATURE GRANULO CYTES PER C MM	MATURE GRANULO CYTES PER C MM	TOTAL DOSE IN MG
1a	300,000	14,280	121,200	109,000	0
	15,000	0	856	7,450	117
1b	100,000	4,330	22,080	62,400	0
	68,000	302	15,410	37,200	49
2	150,000	7,810	72,000	49,500	0
	28,700	287	861	20,900	97
3	47,800	8,560	10,710	24,580	0
	18,850	296	1,059	14,320	97
4	263,000	27,920	98,600	100,800	0
	184,000	6,930	5,550	118,800	86
5	78,000	19,080	8,860	33,300	0
	5,750	39	78	3,921	67

TABLE II LEUCOCYTE COUNTS IN PATIENT WITH LYMPHATIC LEUCEMIA BEFORE AND AFTER TREATMENT

CASE	TOTAL W B C PER C MM	STEM CELLS PER C MM	GRANULO CYTES AND MONOCYTES	LYMPHO CYTES	TOTAL DOSE IN MG
1	102,000	4,270	15,500	82,200	0
	73,800	5,740	260	67,800	132
2	76,400	0	12,500	63,900	0
	77,800	0	8,600	69,200	105
3	35,130	0	4,567	30,563	0
	50,000	0	10,500	39,500	52
4	231,000	26,240	6,644	198,100	0
	180,500	8,570	7,876	164,000	56

Stem cells in both myelogenous and lymphatic leukemia and immature granulocytes in myelogenous leukemia decreased in number, but platelet and erythrocyte counts were variable. Usually mature granulocytes decreased in number during treatment, but lymphocytes of mature type were not affected. Progressive falls in total leucocyte counts occurred for as long as ten days after treatment was stopped.

The most important morphologic effects included the appearance of small numbers of megaloblasts in material aspirated from the sternal marrow. The cells were indistinguishable from those characterizing erythropoiesis in Addisonian pernicious anemia in relapse. They were seen after approximately 100 mg of the medication had been ingested over periods from six to twenty-four days.

In two patients with lymphatic leukemia there were decreases in the sizes of lymph nodes. The hematologic effects of therapy were temporary and never completely adequate because treatment had to be stopped when toxic signs appeared.

The toxic effects, in the order of appearance, included pharyngitis, stomatitis, sore tongue, crampy abdominal pain sometimes associated with diarrhea, dermatitis, and alopecia. Usually pharyngitis first appeared within a few days before or after significant changes in the leucocyte counts were noted. The

total dosage of the drug which produced toxic effects varied from 30 to 75 mg, although one patient received 105 mg without experiencing such signs. There was evidence that when small doses are used initially a larger cumulative dose can be tolerated before toxic symptoms appear.

Autopsies on two patients who expired shortly after therapy was stopped revealed no changes which could be attributed to the medication.

Although 4-amino pteroylglutamic acid had a definite hematologic effect there was no subjective improvement in any of the patients. The use of the drug in chronic leucemia is limited by the early development of toxic phenomena. Further studies with related compounds are warranted.

## 121 EFFECT OF ANTIFOLIC ACID DERIVATIVES ON PATIENTS WITH FAR-ADVANCED CARCINOMATOSIS

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(BY INVITATION) AND GEORGE HASS, M.D., CHICAGO, ILL.

Observations were made on thirty-five patients with far advanced carcinomatosis given pteroylglutamic acid (Teropterin) intramuscularly daily for from two weeks to twelve months. The dose varied from 20 mg daily to 50 mg b.i.d. The following diseases were treated: two epidermoid carcinomas, one carcinoma of the pancreas, three mixed tumors of the parotid, three adenocarcinomas of the cecum, two carcinomas of the colon, five carcinomas of the breast, three transitional cell carcinomas of the bladder, one thymic carcinoma, two bronchogenic carcinomas, one adenocarcinoma of the rectum with carcinoid of the ileum, two carcinomas of the ovary, one epithelioma of the penis, one hypernephroma, one neurofibrosarcoma, one lymphosarcoma, one fibrosarcoma of the orbit, two melanomas, one Ewing's sarcoma, two unclassified malignant bone tumors.

No patients showed any evidence of toxic reactions to the drug even with very large doses. No change in the expected course of the disease was noted in thirty-four of the thirty-five patients given the drug. We were unable to demonstrate any beneficial effects attributable to administration of the drug in these patients.

One patient with pulmonary metastases from a carcinoma of the breast showed roentgenographic evidence of incomplete regression of the metastatic nodules after three months of daily injections of 40 mg pteroylglutamic acid. Though the metastatic nodules persist, there has been no increase in their size five months following cessation of administration of the drug.

Autopsies were performed in twelve instances. No specific effect of the drug on tumors could be demonstrated microscopically.

Twelve patients with malignant disease were given 4-amino pteroylglutamic acid (Aminopterin). The following diseases were treated: one mycosis fungoides, nine carcinomas of the female breast, one adenocarcinoma of the ovary, one melanoma.

Most patients developed ulcerative lesions in the buccal, vaginal or rectal mucosa during therapy. Granulocytopenia occurred in two patients and a fatal pancytopenia developed in one. This patient also had multiple ulcerations of the gastrointestinal tract. Stomatitis and vaginitis usually preceded granulocytopenia. The appearance of toxic reactions frequently necessitated omission

of the drug Stomatitis and leucopenia developed in one elderly patient after two daily injections of 1 mg of 4-amino pteroylglutamic acid

An arrest of rapidly progressive neurological changes and roentgenographic evidence of marked regression in the pulmonary lesions have been noted in one patient with metastatic carcinoma of the breast. Marked regression of regional metastases was noted in one other patient with metastatic mammary cancer. However, both patients experienced severe toxic reactions to the drug.

It is concluded that pteroyltriglutamic acid in the doses given are probably of no value in the treatment of the types of malignancy stated in the foregoing.

No conclusion can yet be drawn on the effect of 4-amino pteroylglutamic acid on malignancy. However, the dramatic response of one patient necessitates further careful observations despite the dangerous toxic side effects.

## 122 EFFECT OF VITAMIN B<sub>12</sub> ON THE HEMATOPOIETIC AND NERVOUS SYSTEMS IN ADDISONIAN PERNICIOUS ANEMIA

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The effect of vitamin B<sub>12</sub> in six patients having classical Addisonian pernicious anemia has been studied. In four of the six patients the diagnosis of pernicious anemia had not been established previously. The remaining two patients had had short courses of treatment with parenteral liver extracts four and three years, respectively, prior to admission to the Clinic. All six patients were in severe relapse. Reticulocyte peaks were noted four to seven days after the institution of therapy and were followed by a rise in erythrocyte counts which reached normal levels in from four to six weeks. Serial aspirations of the bone marrow revealed complete conversion from megaloblastic to normoblastic regeneration in from forty-eight to ninety hours after administration of B<sub>12</sub>. Lantern slides in color will be used to depict the serial changes in the marrow.

Excellent hematologic responses were obtained with doses of 25 µg of vitamin B<sub>12</sub> administered at weekly intervals intramuscularly. Some evidence was obtained to indicate that intervals longer than one week between injections were not always accompanied by a maximal rise in erythrocytes. In one case an initial dose of 6 µg was not followed by a maximal reticulocyte response. An interesting observation was the development of hypochromasia in the erythrocytes of two of the six patients during the period of rise in erythrocyte levels. The significance of this observation is not clearly understood and needs further study.

Four of the six patients had glossitis prior to the institution of therapy. In all four the glossitis improved after treatment was begun and eventually disappeared. Four of the six patients also had definite evidence of subacute combined degeneration of the spinal cord. One patient showed no improvement symptomatically or objectively after receiving 100 µg of vitamin B<sub>12</sub> over a period of sixty-two days. A second patient showed remarkable improvement after receiving 75 µg over a period of thirty-three days. The remaining two patients have not been observed long enough to permit conclusions to be drawn. It should be emphasized, however, that prolonged observation over a period of many months will be necessary before it will be possible to determine whether or not vitamin B<sub>12</sub> therapy will protect permanently the central and peripheral nervous systems in pernicious anemia.

## 123 ESSENTIALITY OF BOTH THE ANTI-PERNICIOUS ANEMIA FACTOR OF LIVER AND PTEROYL-GLUTAMIC ACID FOR HEMATOPOIESIS IN SWINE

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Microcytic anemia with megaloblastic hyperplasia of the marrow was induced in swine by use of a diet essentially free of extrinsic factor and pteroyl glutamic acid (PGA) and containing a crude PGA antagonist and succinyl sulfathiazole.

One pig thus rendered anemic responded incompletely to one dose of PGA free liver extract (15 units) but two months later failed to respond to ten successive daily doses of 15 units each. Four 15 mg doses of PGA then produced an excellent hematologic response.

Another pig failed to respond to five daily doses of 200 Gm of crude casein, used as a source of extrinsic factor. In a second period ten minute daily doses (0.05 mg each) of PGA caused only a small reticulocyte response. In a third period however five daily doses of 200 Gm of crude casein produced a secondary reticulocytosis and a more complete hematologic response.

The findings indicate that a dual deficiency involving PGA and the anti-pernicious anemia (APA) factor was produced. In the first pig it is suggested that the first administration of liver extract produced a small response because PGA was not completely absent while the second administration of liver extract was ineffective because the animal was now devoid of functional PGA. Whether a deficiency of APA factor had also been re-established cannot be stated, but failure to respond to liver extract indicates that the PGA deficiency was, by itself, capable of preventing hematopoiesis. In the second pig it is suggested that extrinsic factor was ineffective until a catalytic amount of PGA was made available.

A third anemic animal responded twice to single doses of 1 mg of PGA given at monthly intervals. However a third dose given after another month failed to elicit a significant response. A single dose of 100 units of purified liver extract produced a good reticulocytosis and a partial hematologic remission. However this restored the ability to respond to PGA as indicated by a good reticulocyte response following a fourth injection of 1 mg.

The results in this animal indicate that deficiencies of PGA and of the APA factor were produced. When the deficiency of APA factor became sufficiently profound PGA was unable to induce hematopoiesis. With removal of the deficiency of the APA factor with a resultant partial remission PGA again became hematopoietically active.

It may be concluded that under the conditions of these experiments neither the APA factor nor PGA can function hematopoietically in the absence of the other and that both substances are essential to the processes involved in the maturation of erythrocytes.

## 124 FURTHER STUDIES ON THE EFFECT OF URETHANE ON LEUCEMIA

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(INTRODUCED BY LOUIS R. LIMARZI, M.D.)

Last year, before this Society, we reported the effect of urethane in leukemia on experience gained with thirty-five cases. We have now studied its effect in a total of eighty-five cases. Of these leukemias 30 were acute, 3 were

acute monocytic (Naegeli type), 2 were subacute myelocytic, 2 were subacute lymphocytic, 34 were chronic lymphocytic, and 12 were chronic myelocytic. One patient with lymphosarcoma of the skin and one with myeloid metaplasia of the spleen were also observed.

In general, our later observations bear out those previously reported. The most consistent results in using urethane may be expected in those individuals having the chronic myelocytic type. The white blood count may be brought to a normal level and, except for minor fluctuations, may be maintained there as long as a proper dose is given. If the drug is stopped, the count steadily and rapidly rises; only to drop again with institution of urethane therapy. The red blood cell and hemoglobin values are improved during this treatment.

In chronic lymphocytic leukemia the results in over 80 per cent of our series are good as concerns the lowering of the white blood count. It appears that in some individuals larger doses than can be tolerated are needed for producing the desired effect.

The results in the use of urethane in acute leukemia are the least striking. Although one observed occasionally what appears initially to be a dramatic fall in the white count, the patient either succumbs at this level or has an equally sudden rise to even higher than pretreatment levels before death. We have observed in two of our patients a temporary complete bone marrow remission to normal. However, this effect was not maintained.

There is little effect of urethane on the size of the lymph nodes or spleen in lymphocytic leukemia. As a matter of fact, in one patient with chronic lymphocytic leukemia the glands actually increased in size. Our usual daily dose has been from  $\frac{1}{2}$  to 3 Gm orally. Some of our patients have been maintained for eighteen months on the drug, with a total of over 5,000 Gm being administered. At this dosage level no toxic effects, other than weight loss, drowsiness and loss of appetite, have been observed in the living. No tissue changes have been observed from the drug as seen at autopsy. Care should be taken in the intravenous administration of urethane as we have observed a fatality when it was given in this manner.

## 125 CLINICAL OBSERVATIONS WITH METHYL-BIS (B-CHLOROETHYL) AMINE HYDROCHLORIDE

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(INTRODUCED BY MAURICE HARDGROVE, M.D.)

Twenty-four patients have been treated with methyl-bis (B-chloroethyl) amine hydrochloride since November, 1946. The recommended course of treatment consisted of 0.1 mg per kilogram of body weight given daily for four days.

There were twelve patients with Hodgkin's disease proved by biopsy. Seven of these patients were x-ray resistant. Four of the group have died. The average duration of life after starting therapy was eight months (range, one to thirteen months). The other three are still living two to six months after treatment. In five additional patients nitrogen mustard was used as the initial form of therapy. In three of these the beneficial effect was short lived. In the other two the results were more satisfactory. One remained in remission for six months and had two courses of the alkyl amine. Later x-ray therapy was instituted. The other has been in complete remission for nine months and has received three courses of therapy. Two of the five have died. Both were given

x ray therapy after failure of response to mustard. The average duration of disease by history in the six patients who have died was 7.4 years (range, six months to twenty years). The average duration of disease in the six living patients is 15 years at present. It appears that the methyl bis (B chloroethyl) amine is of value in treating x ray resistant cases of Hodgkin's disease. It offers no advantages as an initial form of therapy.

Two patients with lymphosarcoma and two with giant follicle lymphoma were given nitrogen mustard. One of each of these had a partial, unsatisfactory remission. One in each category is still in remission nine months after a single course.

One patient with chronic lymphocytic leucemia, one with chronic myelocytic leucemia, and one with polycythemia vera were treated with partial and brief response.

Two patients with squamous cell bronchiogenic and one with small cell adenocarcinoma of the lung, one with squamous cell carcinoma of the floor of the mouth, and one with malignant myeloma of the choroid were treated with but slight effect.

## 126 THE USE OF RADIOARSENIC ( $As^{76}$ ) IN THE TREATMENT OF LEUCEMIA AND ALLIED DISORDERS OF THE HEMATOPOIETIC TISSUES

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Nineteen patients with tumors of the hematopoietic system have been given  $As^{76}$  prepared by pile irradiation of arsenic trioxide and cacodylic acid. Arsenic  $^{76}$  has a half life of 26.8 hours and energetic beta and gamma radiations. Doses of  $As^{76}$  ranged from 1 to 80 millicuries with 3 to 10 mg. of stable arsenic and were administered intravenously as the trioxide. Preliminary studies indicated that each millicurie was approximately equivalent to one roentgen of total body radiation. In four previously untreated patients with chronic myelocytic leucemia, single injections of this radioisotope produced clinical and hematologic remissions. One of these patients is still in remission after eleven months. However, no significant clinical response was produced by the isotope in two patients with chronic myelocytic leucemia who no longer responded to conventional irradiation therapy. One patient with an acute myelocytic leucemia failed to respond. One patient with a subacute myelocytic leucemia had clinical and hematologic remissions of about two months after each of two injections. However, this latter case required frequent transfusions and an osteomyelitis of the femur failed to heal in spite of chemotherapy and surgery.

Three patients with chronic lymphocytic leucemia treated with less than 30 millicuries were not benefited. One aged male previously insensitive to nitrogen mustard and needing massive transfusions responded with a decrease in lymph node enlargement and splenomegaly, an increase in subjective sense of well being as well as a depression of the white count for about two months. Two patients with acute lymphocytic leucemia were treated with 70 and 60 millicuries, respectively. One had a temporary and incomplete remission; in the other, a definite clinical and hematologic remission of approximately eight weeks occurred.

Two patients with polycythemia rubra vera were treated with radioarsenic. A relatively brief remission was produced in one of these patients who was given a dose of 45 millicuries. The other patient received essentially a trace

dose Four patients with Hodgkin's disease received less than 13 millicuries of  $As^{76}$  each The clinical effect of the isotope on the course of these four patients was essentially insignificant Likewise, two patients with multiple myeloma failed to respond favorably to this radioisotope

The huge liver of a patient with metastases from a carcinoma of the stomach decreased about 5 cm in size following 80 millicuries of  $As^{76}$  in two doses Nevertheless, his clinical condition continued to deteriorate and he died within two weeks

## 127 A METHOD OF FREQUENT ESTIMATIONS OF CIRCULATING PLASMA VOLUME WITH ERYTHROCYTE COUNTS, ADAPTABLE TO LONG-CONTINUED OBSERVATIONS

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The fluctuations in plasma volume may be studied at will over a period of many days with little inconvenience to the subject An initial measurement of the plasma volume by Evans blue dye (T-1824) and a coincident erythrocyte count are correlated with subsequent changes in the peripheral erythrocyte counts The proof depends upon the demonstration of the following facts (1) With careful attention to the usual technique for counting erythrocytes, values were obtained with a standard deviation of  $\pm 68,000$  cells per cubic millimeter (2) Erythrocyte counts on the peripheral blood of normal active adults were found to vary from day to day, sometimes with a range of 1,000,000 cells per cubic millimeter The variation frequently occurred in cycles of two or three days (3) In subjects who had received transfusions of blood of heterologous group, the unagglutinable cell counts (donor's blood) varied coincidentally with those of the recipient, in the same direction and in approximately the same magnitude (4) When estimates of the plasma volume were made from the cell count some days after the original measurement by the dye method, they were found to correspond with a second measurement by the dye within  $\pm 5$  per cent

The method consists of an initial measurement of the circulating plasma volume by Evans blue dye coincident with an erythrocyte count The fluctuations in erythrocyte counts thereafter can be observed over a period of many days and the plasma volume calculated from any single observation by the equation

$$PV_2 = \frac{C_1 \times PV_1}{C_2}$$

where  $PV_2$  is the calculated plasma volume in milliliters,  $PV_1$  is the plasma volume in milliliters measured by the dye method,  $C_1$  is the erythrocyte count in millions per cubic millimeter in the peripheral blood taken at the time when  $PV_1$  was determined, and  $C_2$  the erythrocyte count in millions per cubic millimeter at the time of the calculation

The method assumes (1) that the total number of red cells in the body remains the same and (2) that the hematocrit of the peripheral blood bears a constant relationship to the total body hematocrit

This procedure has been employed to follow the daily fluctuations in plasma volume during normal activity, during hydration and dehydration, and in recipients undergoing blood transfusion The same method is applicable to the study of many problems in the clinic Daily erythrocyte counts alone, when accurately performed, should serve as an indication of variations in plasma



volume in patients who are not losing blood and whose bone marrow is functioning normally

## 128 THE INFLUENCE OF RUTIN BENADRYL AND PROTAMINE ON EXPERIMENTAL PURPURA IN THE GUINEA PIG

FRANK H BELFUS, M D (BY INVITATION) AND FREDERICK W MADISON M D  
MILWAUKEE WIS

Thrombocytopenic purpura was produced in eighty guinea pigs by the use of antiplatelet serum according to the method of Bedson. The purpuric animals were divided into four groups of twenty each. One group served as control and received only the previously prepared antiplatelet serum. A second group received rutin, a third group Benadryl and a fourth group Protamine simultaneously with the serum. Serial determinations of erythrocytes, platelets and petechiae were made over a twenty four hour period following the administration of the serum and the drug. The petechial responses were measured by means of a negative pressure apparatus and the petechiae counted within a standard area.

The animals of the control group which received only antiplatelet serum exhibited a slight drop in erythrocytes, a marked fall in the platelets and a sharp rise in petechial formation. The greatest number of petechiae occurred within six hours after the injection of antiplatelet serum which was well in advance of the maximum fall of platelets. When the platelets had reached their lowest numbers the petechial response had subsided.

The second group of animals were given rutin in repeated injections of 10 mg per kilogram of body weight in addition to the initial dose of antiplatelet serum. These animals exhibited the same trend in platelet depression as the control group. The petechial response of the rutin group was approximately 60 per cent less than that of the control group.

The third group of animals were given the antihistaminic drug, Benadryl in doses of 10 mg per kilogram in addition to the antiplatelet serum and a similar platelet depression occurred. However, the petechial response showed reduction of about 40 per cent as compared with the control animals.

The fourth group of animals received protamine in repeated doses of 2 mg per kilogram in addition to antiplatelet serum. Again the platelet depression followed the usual pattern but the petechial response was markedly diminished. Almost 90 per cent fewer petechiae were produced than in the control group.

Each of the drugs tested therefore showed ability to alter the vascular response (in varying degrees) under the conditions of this experiment but none showed any effect on the thrombocytopenia.

## 129 EFFECT OF VITAMIN K ON DICUMAROL INDUCED HYPOPROTHROMBINEMIA IN RATS

E J BOYD M D (BY INVITATION) AND E D WARNER, M D  
IOWA CITY IOWA

Most of the reports dealing with the effect of vitamin K on Dicumarol induced hypoprothrombinemia have been based on one or another of the one stage methods for prothrombin estimation. It has been shown that these methods depend not only on the concentration of prothrombin but also on the rate of conversion of prothrombin to thrombin. The two stage method of prothrombin determination separates the conversion phase from the clotting phase and thereby largely eliminates the rate of conversion of prothrombin to

thrombin as a factor in prothrombin measurement. For this reason, it seemed pertinent to study the effect of vitamin K on Dicumarol-induced hypoprothrombinemia by the two stage prothrombin method.

Albino rats were made hypoprothrombinemic by the administration of Dicumarol. To control the dosage better, the Dicumarol was given by stomach tube. Vitamin K was given in daily doses as menadione in oil by stomach tube, or as Hykinone by intraperitoneal injection. Doses as large as 50 mg of menadione and 4 cc Hykinone (1 cc equivalent to 25 mg menadione) per day were used. The data obtained by the two-stage prothrombin determination indicate that (1) rats tend to develop a tolerance to Dicumarol, (2) massive doses of vitamin K in the form of menadione, or Hykinone, have no definite effect on Dicumarol-induced hypoprothrombinemia in rats, from either the prophylactic or the curative standpoint.

### 130 THE USE OF INTRAMUSCULAR HEPARIN FOR ANTICOAGULANT THERAPY

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CHICAGO, ILL

In a series of 100 patients, subcutaneous and intramuscular injections of heparin were given to maintain a prolonged coagulation time for prophylactic or therapeutic purposes. The solutions used were 1 and 10 per cent aqueous heparin and a preparation which uses gelatin dextrose and vasoconstrictors to delay absorption.

After considerable trial with various dosage and timing, the following tentative schedule has been developed.

The patient first is given 1 cc of 1 per cent heparin intravenously to determine his heparin tolerance. The coagulation time is determined before and only once, ten minutes, after the injection. If there is a 100 per cent increase in coagulation time following the injection, he is regarded as having a normal response and is given 5 cc of heparin intravenously. One hour following this injection, which usually raises coagulation time to about 15 minutes by the venous method, the first intramuscular injection is given with the heparin glycerin mixture. In from twelve to sixteen hours this is repeated, the coagulation time by the capillary method is always done before the next injection to avoid a stepladder type of effect.

There has been no reaction to the intramuscular injections either in the form of hematoma or induration, and the coagulation times have shown a stable level which was hitherto impossible to obtain even by the continuous intravenous drip.

### 131 THROMBOCYTOPENIA SECONDARY TO INFECTIOUS MONONUCLEOSIS

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CHICAGO, ILL

Thrombocytopenic purpura is a rare complication of infectious mononucleosis. A report is made of a 19-year-old white female student nurse who developed petechiae, ecchymoses, and menorrhagia during the course of infectious mononucleosis. The platelet count fell to 10,000 per cubic millimeter. Spontaneous recovery accompanied convalescence from the infectious mononucleosis. The patient has remained well with a normal platelet count and no further bleeding episodes.

A review of the literature reveals only two identical cases. In two other reported instances there appeared to be a pre-existing bleeding diathesis which was aggravated by the infectious mononucleosis. In other possible cases the diagnosis of infectious mononucleosis was never definitely established or in sufficient details were reported to permit comparison.

Although the platelet count is said to be normal in infectious mononucleosis, no report of serial counts in this disease was found. We have determined the number of platelets at frequent intervals in seven consecutive cases of infectious mononucleosis. In five of the seven patients the platelet count was below the lower limit of normal during the first week of the disease. In one case the platelets were just above the lower limit of normal, and in another, initial counts were not made until the second week of the disease. During the second week of the illness a mild thrombocytosis varying from one and one-half to five times the lowest count occurred in six patients. This was followed by a gradual return to normal values. The case studied first in the second week of the disease, showed a mild thrombocytosis which subsequently returned to normal. In four of the seven patients the spleen was palpable and abnormal liver function was present in all.

The possibility that thrombocytopenic purpura in infectious mononucleosis may result from a similar but exaggerated depression of the blood platelets is suggested.

The possible roles of infection and hypersplenism as a cause of the thrombocytopenia will be discussed.

## 132 THE EFFECT OF DIGITALIS ON THE COAGULABILITY OF BLOOD

JOHN HASLOP FLINN M.D. ROCHESTER MINN.

(INTRODUCED BY HOWARD B. BURCHELL M.D.)

The high incidence of thromboembolic complications in cases of congestive heart failure is generally recognized. In the past five years several reports have appeared in the literature which implicated digitalis as a contributing factor to intravascular coagulation of the blood. Other reports have subsequently appeared which seem to deny this implication. The present study was undertaken in an attempt to determine whether certain cardiac glucosides do change the coagulability of the blood and if so how these changes can be measured. Because of the controversial reports it seemed evident that an investigation which would include both animal and clinical research and would consist of a study of blood coagulation by several methods before and during digitalization was indicated.

The problem was first investigated in the physiology laboratories of the Mayo Clinic and rabbits and rats were used. The study was completed in the Worrall Hospital, Rochester, Minn. with patients on the cardiac services of the Mayo Clinic. Studies of coagulation included the Lee-White coagulation time, prothrombin times of whole and diluted plasma and heparin retarded coagulation time.

Results of the investigation show that lanatosid C and digitoxin administered to normal rabbits and rats in doses simulating therapeutic use of the drugs have a definite accelerating effect upon coagulation of the blood as measured by the Lee-White coagulation time tests. In rabbits the *in vitro* heparin retarded coagulation time test showed a similar effect on coagulation of the blood. No effect was noted on the prothrombin time of whole or diluted plasma. In the normal rabbit relatively large doses of lanatosid C appear to have an antagonistic effect to Dicumarol.

The blood of clinically digitalized patients was not changed from that of normal subjects as measured by the Lee-White coagulation time tests or by the prothrombin time studies of whole or diluted plasma. However, digitalization appeared to diminish the anticoagulant effect of heparin in the human being.

These results seem to indicate that digitalis may be a factor in promoting intravascular coagulation of blood.

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## EDITORIAL ANNOUNCEMENT

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On January 1, 1949, Doctor Clayton G. Loosli will become Editor of the JOURNAL OF LABORATORY AND CLINICAL MEDICINE. After that date, all correspondence relating to editorial matters should be addressed to him at the University of Chicago, Department of Medicine.

For the past two years, this Journal has been published under the editorial direction of the Central Society for Clinical Research. It seems appropriate now to announce the way in which the Editor and the members of the Board of Editors are selected. The Editor is appointed by the Council of the Central Society for a term of three years, he may be appointed for a second three-year term, but may not serve consecutively for longer than six years. When the Editorship is to be vacated for any reason, the Board of Editors recommends a successor from the Society's membership to the Council. The Council may accept this recommendation or may make its own appointment. Doctor Loosli represents the unanimous and enthusiastic choice of the Council, the Board of Editors, and the retiring Editor.

Members of the Board of Editors are appointed by the Editor to serve for a three-year period. Each member is eligible for reappointment to a second, but not to a third consecutive term. Appointments are staggered so that four or five terminate at the end of each calendar year. Members of the Board are selected from among the active, emeritus, and adjunct membership of the Central Society for Clinical Research.

The retiring Editor wishes to express publicly his sincere appreciation first, to all members of the various Editorial Boards who have served with him during the past five years, second, to the many clinical investigators who have supported the Journal by sending their manuscripts to it, and third, to the editorial department of The C. V. Mosby Company for constant, wholehearted cooperation.

# INDEX TO VOLUME 33

## AUTHORS INDEX

In the index following the author's name the title of the subject is given as it appeared in the JOURNAL

### A

- ABRAMSON DAVID I, COLL WARREN H  
KEETON ROBERT W GEPHARDT,  
MAURICE DANYIEWICZ JOSEPHINE M  
PRESLEY, SOPHIE J, AND LAYERS  
GEORGE Influence of physical fit-  
ness and convalescence from major  
surgical procedures on the magnitude  
of the responses to exercise of the  
blood and urine lactate 1474
- ADAMS WILLIAM S (See LAWRENCE VALEN-  
TINE AND ADAMS) 1077
- ALBANESE ANTHONY A AND LEIN MARILYN  
The microcalorimetric determination  
of sodium in human biologic fluids  
46
- ALLEN J GARROTT (See JACOBSON MARKS  
GASTON ALLEN AND BLOCK) 1000
- JACOBSON LEON O AND GROSSMAN BUR-  
TON J A study of the plasma de-  
fect in patients whose bleeding is  
temporarily controlled by protamine  
sulfate or toluidine blue 1480
- ALPER CARL (See CHOW HAIL DUFFY  
AND ALPER) 1440
- ALT, HOWARD L (See ANGLE AND ALT)  
1652
- (See PIERCE AND ALT), 1642
- ANDERSON HAMILTON H (See FARBER  
EAGLE, ANDERSON, AND GORMAN)  
99
- ANGLE, RICHARD M AND ALT HOWARD L  
Thrombocytopenia secondary to in-  
fectious mononucleosis 1652
- ARNOLD, W M JR, AND WEAVER R H  
Quick microtechniques for the identi-  
fication of cultures I Indole pro-  
duction 1334
- ASSALI N S (See BRIST ASSALI, AND  
FEIRIS) 1466
- AUELROD ARNOLD R (See BERMAN, AUEL-  
ROD JACOBSON MORAN VONDER-  
HEIDE AND SHARP) 1625
- (See BERMAN, AUELROD VONDERHEIDE  
AND SHARP), 1613

### B

- BALDES, EDWARD J (See BERRY, BALDES,  
ESSEX AND WATKIN) 101
- BALKIN SELMOUT S, AND GOOTWICK ABRA-  
HAM The effects of diazepamol on  
the electrocardiogram 972
- BAKER NELSON W (See ROSENBAUM AND  
BAKER), 142

- BARNES MILDRED W (See FINLAND, BARNES,  
MEADS AND ORY) 15
- (See FINLAND ORY MEADS, AND  
BARNES) 32
- (See MORGAN BARNES, AND FINLAND),  
1212
- MORGAN HERBERT R AND FINLAND MAX  
WELL Isolation and identification  
of influenza viruses during the epi-  
demic of December 1945 309
- BARNETT GWENDOLYN (See LOOSLI SCHOEN-  
BERGER AND BARNETT) 789
- BARNETT VOLHAMME H (See HOWITT AND  
BARNETT) 1402
- BARTLETT WILLARD, JR. Pyloric balance in  
man: concept and application, 1623
- BECK ELIOT M AND MUNTZ HASCALL H  
Experimental therapy of generalized  
torulo is in rats with streptomycin,  
1154
- BEDINGER PAUL L Further studies on the  
effect of urethane on leucemia 1647
- BEHRMAN VIVIAN G (See GOODRICH  
BEHRMAN AND HARTMAN) 1454
- BELFUS FRANK H AND MADISON FREDERICK  
W The influence of rutin benadryl  
and protamine on experimental pur-  
pura in the guinea pig 1601
- BENDITT EARL P HUMPHREYS, ELEANOR M  
WISSLER ROBERT W STEFFEE C  
HAROLD FLAZIER, LAURENCE E AND  
CANNON PAUL R The dynamics of  
protein metabolism I The inter-  
relationship between protein and  
caloric intakes and their influence  
upon the utilization of ingested  
protein for tissue synthesis by the  
adult protein depleted rat, 257
- WOOLRIDGE ROBERT L AND STEPTO,  
ROBERT The dynamics of protein  
metabolism II The relationship  
between the level of protein intake  
and the rate of protein utilization  
by protein depleted men and rats,  
269
- BENNETT ALENE (See MAY, BENNETT, LANE  
FUTCH SCHOOMER AND GREGORY)  
1494
- BENNETT W A (See SPRAGUE HAYLES,  
MASON POWER, AND BENNETT),  
1412
- BENOTTI JOSEPH (See NOTHMAN PRATT  
AND BENOTTI) 83
- BERGMAN H C (See PRINZMETAL, KPLUGER,  
AND BERGMAN), 497

- , KRUGER, H E, AND PINZMETAL, MYRON  
The mechanism of delayed death  
following thermal trauma, 506
- BERGMANN, MARTIN (See SHATZ, BERGMANN, AND GRAY), 1588
- BERKMAN, SAM (See HENRY, KIRKWOOD, BERKMAN, HOUSEWRIGHT, AND HENRY), 241
- BERMAN, BERNARD Electrocardiographic changes following meals in patients with angina pectoris, 1501
- BERMAN, JEPOME R (See HAMBURGER, BERMAN, AND FABRIZIO), 1460
- BERMAN, LAWRENCE, ADELFOB, ARNOLD R, JACOBSON, SAMUEL D, HORAN, THOMAS N, VONDERHEIDE, ELMORE C, AND SHARP, ELWOOD A The blood and bone marrow in patients with cirrhosis of the liver, 1628
- , —, VONDERHEIDE, ELMORE C, AND SHARP, ELWOOD A Treatment of chronic leucemia with folic acid antagonist, 1643
- BERNAT, LEO ALLEN (See HEMINGWAY, BERNAT, AND MASCHMEYER), 126
- BERNSTEIN, ARTHUR (See PAPSONNET, BERNSTEIN, KLOSK, HIRSCHBERG, RUBIN, AND PIRK), 602
- (See SWARTS, BERNSTEIN, REAGINS, AND MEYER), 1633
- BERNSTEIN, THEODORE B (See FEINBERG AND BERNSTEIN), 319
- BERNSTEIN, THEODORE C (See SILBERSTEIN, BERNSTEIN, AND STERN), 1204
- BERNY, MAXWELL R, BALDES, EDWARD J, ESSEX, HIRAM E, AND WAKIM, KHALIL G A compensating plethysmograph for measuring blood flow in human extremities, 101
- BERTRAND, J J, WAYNE, H, AND TOBIAS, C A Distribution of gold in the animal body in relation to arthritis, 1133
- BETHELL, FRANK H, MEYERS, MURIEL C, AND NELIGH, ROSALIE B Vitamin B<sub>12</sub> in pernicious anemia and puerperal macrocytic anemia, 1477
- BLANKENHORN, DAVID, AND WOLFF, HAROLD G Myelitis following the administration of neoparsphenamine, 1165
- BLITZ, DIANA (See SPICER AND BLITZ), 417
- BLOCK, MATTHEW H (See JACOBSON, MARKS, GASTON, ALLEN, AND BLOCK), 1566
- , JACOBSON, LEON O, NEAL, WILLIAM B, JR., AND SMITH, TAYLOR R The use of radioarsenic (As<sup>76</sup>) in the treatment of leucemia and allied disorders of the hematopoietic tissues, 1649
- BLOCK, WALTER D (See ROBINSON, CONN, BLOCK, AND LOUIS), 1473
- , GEIB, NAOMI C, AND ROBINSON, WILLIAM D Metabolism, toxicity, and manner of action of gold compounds in the treatment of arthritis VIII The effect of BAL and other thiol compounds in preventing the inhibition of oxygen consumption of rat tissues produced by gold salts, 1381
- BOBB, J R R (See WALLACE, LITTLE, AND BOBB), 845
- BOGEF, WILLIAM P, MILLER, A KATHERINE, TILLSON, ELIZABETH, AND SHAFER, GRACE A Caronamide plasma concentrations, urinary recoveries, and dosage, 297
- BOHLS, S W, AND SHAW, PHYLLIS An increase of complement units by the use of egg albumin, 239
- BOLLMAN, JESSE L (See SALASSA, BOLLMAN, AND DRY), 1393
- (With the technical assistance of VAN HOOK, EMERY) A cage which limits the activity of rats, 1348
- , CAIN, JAMES C, AND GRINDLAY, JOHN H (With the technical assistance of VAN HOOK, EMERY) Techniques for the collection of lymph from the liver, small intestine, or thoracic duct of the rat, 1349
- BORDEN, CRAIG, WILSON, RUSSELL H, AND EBERT, RICHARD V Study of pulmonary hypertension in chronic hypertrophic pulmonary emphysema, 1453
- BOFG, JOSEPH F, AND CRAIG, DAVID M Experiences with a mercurial diuretic for subcutaneous injection, 1635
- BOYD, E J, AND WARNER, E D Effect of vitamin K on dicumarol induced hypoprothrombinemia in rats, 1431, 1651
- BOYD, JULIAN D, CHEYNE, V D, AND WESSELS, K E Correlation of lactobacillus counts with extent of dental caries in an institutional population, 1614
- BOYD, LINA J (See MCGAVACK, WEISSBERG, AND BOYD), 595
- BRADBURY, J T (See BROWN, BRADBURY, AND KRAUSHAAR), 1465
- (See KRAUSHAAR AND BRADBURY), 1195
- BRADEN, EDWIN S, JR (See TURNBULL AND BRADEN), 1624
- BIAUDE, ABRAHAM I, AND GOLD, DAVID Antibody formation in human subjects following the ingestion of heat killed Brucella abortus, 1459
- BRAUNSTEIN, JOHN (See MCGUIFE, GREEN, COUTER, NOETKAER, IGLEH, HAUENSTEFIN, AND BRAUNSTEIN), 1457
- BRENDMOEN, C (See BRENDMOEN AND BRENDMOEN), 1089
- BRENDMOEN, O J, AND BRENDMOEN, C Anti A and Anti B isogglutinin titers in Rh immunized pregnant women, 1089
- BROWN, BARBARA B, AND WERNER, HAROLD W (With the technical assistance of PETERS, EMMETT L) The pharmacologic properties of 2[a(2 dimethylaminoethoxy) α methyl

- benzyl] pyridine succinate a new antihistaminic agent 329
- BROWN, HUGH S (See SLAUGHTER BROWN AND WARREN), 743
- BROWN RAY K READ JOHN T WISEMAN BRUCE K AND FRANCE, WESLEY G The electrophoretic analysis of serum proteins of the blood by crasmas 1523
- BROWN, WILLIS E BRADBURY J T AND KARLSHAAR, OTTO F Effect of sedatives on urinary volume of pregnant women 1465
- BRYET ALBERT A ASSALI, N S, AND FEPRIE EUGENE B Evaluation of neurogenic and humoral factors in blood pressure maintenance in normal and toxemic pregnancy using tetraethyl ammonium chloride 1466
- BRYAN A HUGHES WILLIAMS T FRANKLIN AND WEBB CELIA R The determination of hemoglobin as pyridine hemochromogen 1638
- BUCK R E Observations on alcoholic fatty liver the use of interval needle biopsy and liver function tests 555
- BUEBING, ERNEST (See MERFEL AND BUEBING), 371
- BURANTZ SAMUEL C DAMMUS GUSTAV J WILSON KEITH S AND JOHNSON MARY C The inhibitory effect of nitrogen mustard (bis beta chloro ethyl amine) on the development of humoral antibodies cutaneous hypersensitiveness and vascular lesions in rabbits following injections of horse serum 1463
- C
- CAIN JAMES C (See BOLLMAN CAIN AND GRINDLAY), 1449
- CALLEBAUT C FELDMAN V JR ROBBARD S AND KATZ L N The effects of acute hypoxia on the sensitivity of the heart to acetylcholine 1499
- CALLEY IRWIN R AND FISCHER JACK W A comparative evaluation of extremity and precordial leads in the diagnosis of acute myocardial infarction 1500
- AND KEETON ROBERT W The electrocardiographic response to the anoxemia test in patients with hyperthyroidism 1500
- CALVARY E (See WOLFSON COHN CALVARY AND THOMAS), 1276
- CAMPBELL CHARLOTTE C (See SASLAW AND CAMPBELL), 811
- AND SASLAW SAMUEL (With the assistance of KNIBB SHIRLEY A) The use of yeast phase antigens in a complement fixation test for histoplasmosis II Results with ground antigens, 1207
- CAMPBELL D C (See HALL AND CAMPBELL), 1616
- CANNON, PAUL R (See BENDITT, HUMPHREYS WISSELER, STEFFEE, FRAZIER, AND CANNON), 257
- CARTER GEORGE H (See LARCOM AND CARTER), 875
- CARTWRIGHT GEORGE E FAY JANE, TATTING, BETTY AND WINTROBE MAXWELL M Pteroylglutamic acid deficiency in swine effects of treatment with pteroylglutamic acid liver extract, and protein 397
- CASON LOUISE R (See MCBURNEY AND CASON), 1037
- CASTLE, WILLIAM B (See DALAND AND CASTLE), 1082
- (See WAGLEY SHEN, GARDNER, AND CASTLE), 1197
- CHANG NAI CH'U AND HOU TSUNG CHANG Cold hemagglutinin test by a slide method 1447
- CHAMMAN DON W AND GUGLE LLOYD J Intravenous catheterization of the heart in the diagnosis of congenital heart disease 1489
- CHARLES CECIL M (See EDWARDS CHARLES AND MACBRIDE), 1615
- CHERTACK MELVIN M (See GRIMELLI, CHERTACK RHETTA KENDRICK AND FORREST), 1617
- GRIMELLI LUKE J RHETTA HELEN L AND KEETON ROBERT W Effect of insulin on tubular reabsorption of glucose in diabetic patients 1616
- CHEYNE A D (See BOAD, CHEYNE AND WISSELS), 1614
- CHIFFELLE THOMAS R (See HAGEMANN AND CHIFFELLE), 573
- CHILDS D S JR KEATING F R JR WILLIAMS M M D AND POWELL M H The effect of varying the dose of iodine on the behavior of radioiodine tracers in patients with exophthalmic goiter 1619
- (HOW BACON F, AND DEBIASE SHIRLEY The effect of oral administration of casein hydrolysate on the total circulating plasma proteins of man, 493
- HALL LOIS DUFFY B J AND ALPER CARL A micromethod for the determination of the human albumin globulin and hemoglobin contents 1440
- HOMBERGER F DEBIASE SHIRLEY AND PETERMANN MARY L A clinical method for the determination of human albumin by means of a precipitin reaction 1052
- CHRISTY HAROLD W (See LEROY NALFSAI, AND CHRISTY), 1496
- CISNEROS F (See MILLS CISNEROS AND KATZ), 1488
- CHURCH MARY C (See HARVED CUNNINGHAM HALLIDAY VESSEY YODA CHURCH, HINE COSGROVE AND SUBBA POW), 216
- CHURCH ROBERT W (See TALSO CROSBY AND CLAFKE), 430

- CLEMENTS, ALFRED B (*See* KIBRICK AND CLEMENTS), 662
- CLEVELAND, F (*See* COGSWELL, CLEVELAND, GALL, WEISBROD, AND SCHIFF), 1627
- COATNEY, G ROBERT (*See* KENT, CULWELL, COATNEY, AND COOPER), 747
- CODE, CHARLES F (*See* KEATING AND CODE), 1609
- (*See* MOPLOCK, FERRIORMI, AND CODE), 1522
- COGSWELL, R C, CLEVELAND, F, GALL, E A, WEISBROD, F, AND SCHIFF, L Experiences with needle biopsy of the liver in hepatic cirrhosis, 1627
- COHEN, PHILIP P, AND THOMPSON, FRANCES L A comparative study of micro and macroelectrophoretic analysis of human and rat serum, 75
- COHN, CLARENCE (*See* WOLFSON, COHN, CALVARY, AND THOMAS), 1276
- AND WOLFSON, WILLIAM QUITMAN Studies in serum proteins II A rapid clinical method for the accurate determination of albumin and globulin in serum or plasma, 367
- COLE, WARREN H (*See* ABRAMSON, COLE, KEETON, GEPHARDT, DYNIEWICZ, PRESLEY, AND LAVERS), 1474
- COLLENTINE, GEORGE E On the efficacy and safety of glycine administered by vein, 1555
- COLLINS, HARVEY S, KRAFT, LISBETH M, KINNEY, THOMAS D, DAVIDSON, CHARLES S, YOUNG, JOHN, AND STARE, FREDRICK J Parenteral nutrition III Studies on the tolerance of dogs to intravenous administration of fat emulsions, 143
- CONN, JEROME W (*See* ROBINSON, CONN, BLOCK, AND LOUIS), 1473
- , LOUIS, LAWRENCE H, AND WHEELER, CLAYTON E Production of temporary diabetes mellitus in man with pituitary adrenocorticotrophic hormone, relation to uric acid metabolism, 651
- COOKSEY, WARREN B The relief of edema in nephrosis by plasma albumin and gelatin infusions, 1491
- COOPER, TALBERT (*See* STICKNEY, HAGEDORN, MILLS, AND COOPER), 1481
- COOPER, W CLARK (*See* KENT, CULWELL, COATNEY, AND COOPER), 747
- CORCORAN, A C (*See* MASSON, CORCORAN, AND PAGE), 1489
- (*See* TAYLOR, CORCORAN, FERTIG, AND PAGE), 1469
- AND PAGE, IRVINE H Methods for the chemical determination of corticosteroids in urine and plasma, 1326
- CORRELL, HOWARD L (*See* LINDERT AND CORRELL), 1502
- COSGROVE, RACHEL (*See* HARNED, CUNNINGHAM, HALLIDAY, VESSEY, YUDA, CLARK, HINE, COSGROVE, AND SUBBAROW), 216
- COURTER, SANFORD R (*See* MCGUIRE, GREEN, COURTER, NOERTKER, IGLAUER, HAUENSTEIN, AND BRAUNSTEIN), 1457
- COWIE, DEAN B (*See* SMITH, STEELE, EAKIN, AND COWIE), 635
- CRAIG, DAVID M (*See* BORG AND CRAIG), 1635
- CRESSON, SAMUEL L, AND GLENN, WILLIAM W L Venous catheterization with polyethylene tubing, 1597
- CRITCHFIELD, FRANCES H (*See* MOSES, CRITCHFIELD, AND THOMAS), 448
- CROSLEY, ARCHER P, JR. (*See* TALSO, CROSLEY, AND CLARKE), 430
- CRUMLINE, JOSEPH (*See* TAYLOR, SLAUGHTER, PRESTON, CRUMPINE, AND HASS), 1645
- CULWELL, WILLIAM B (*See* KENT, CULWELL, COATNEY, AND COOPER), 747
- CUNNINGHAM, RAYMOND W (*See* HARNED, CUNNINGHAM, HALLIDAY, VESSEY, YUDA, CLARK, HINE, COSGROVE, AND SUBBAROW), 216
- CURRY, JOHN J, AND LEARD, SAMUEL E The action of pilocarpin on the lungs in normal and asthmatic subjects, 585
- CUSICK, GEORGE (*See* GREEN, JOHNSON, LOBB, AND CUSICK), 332
- D
- DALAND, GENEVA A, AND CASTLE, WILLIAM B A simple and rapid method for demonstrating sickling of the red blood cells the use of reducing agents, 1082
- DAMMIN, GUSTAVE J (*See* BUKANTZ, DAMMIN, WILSON, AND JOHNSON), 1463
- DARLING, DOROTHY B (*See* METCOFF, DARLING, SCANLON, AND STARE), 47
- DAVIDSON, CHARLES S (*See* COLLINS, KRAFT, KINNEY, DAVIDSON, YOUNG, AND STARE), 143
- DAVIS, W D, JR., SEGALOFF, ALBERT, AND JACOBS, WILLIAM The effect of desoxycorticosterone acetate and propylene glycol in experimental renal hypertension, 1483
- DEBIASE, SHIRLEY (*See* CHOW AND DEBIASE), 453
- (*See* CHOW, HOMBERGER, DEBIASE, AND PETERMANN), 1052
- DEGOWIN, ELMER L (*See* HAMILTON, SHEETS, AND DEGOWIN), 1650
- DE TAKATS, GEZA, AND LASNER, JACOB The insulin tolerance of the hypertensive patient, 1487
- AND ROGERS, EVELYN M The use of intramuscular heparin for anticoagulant therapy, 1652
- DEY, F L (*See* DOLKART, HALPERN, KUTZ, LESH, WOLNIAK, AND DEY), 1608
- DIECKMANN, WILLIAM J, PRIDDLE, HAROLD D, TURNER, ROSE, AND TREPTOW, BETTY Anemia of pregnancy treated with molybdenum iron complex, 1641
- DILLE, ROGER S, AND WATKINS, CHARLES H A method for the determination of plasma catalase and the values obtained in normal adults, 480
- AND — Plasma catalase in hemolytic diseases and other abnormal state, 487



- DOAN, CHARLES A (See WRIGHT, DOAN DODD, AND THOMAS) 708  
 — (See ZANES, DOAN AND HOSTER), 1002  
 DODD, VERNE A (See WRIGHT, DOAN, DODD AND THOMAS) 708  
 DOLKART, R E HALPERN, B KUTZ A L LESH J B, WOLMAN, B AND DEY F L The role of the plasma protein fractions in the action and transport of penicillin 1608  
 DONOHUE, WILLIAM L AND FREMBS I ARTHUR Maternal isoimmunization without evidence of clinical erythroblastosis fetalis in the newborn 526  
 DOWLING, HARRY F (See ROBINSON, HENSH MILLOFF, AND DOWLING) 1232  
 DOYLE, WILLIAM M (See EVANDER AND DOYLE) 869  
 DREYFUSS, FRITZ A dilution turbidity test in the serum in comparison with the thymol turbidity and cephalin cholesterol flocculation tests 672  
 DRY, THOMAS J (See SALASSA, BOLLMAN AND DRY), 1393  
 DUBIN, ALVIN (See POPPER, STEIGMANN, DIWIEWICZ AND DUBIN) 1630 1631  
 DUFFY, A T (See CHOW, HALL, DUFFY AND ALPER) 1440  
 DUVN, RAYBURN W Recovery and estimation of radioactive isotopes from biologic tissues I Gold 1169  
 DIWIEWICZ, HATTIE (See POPPER, STEIGMANN, DIWIEWICZ AND DUBIN) 1630 1631  
 DIWIEWICZ, JOSEPHINE M (See ABRAMSON, COLE, KEETOV, GEPHARDT, DIWIEWICZ, AND LAVERS) 1474
- E
- EAGLE, HENRY R (See FARBER, EAGLE AND ANDERSON AND GORMAN) 799  
 EAKIN, ROBERT E (See SMITH, STEELE, EAKIN AND COWIE) 635  
 EBERT, RICHARD V (See BORDEN, WILSON AND EBERT), 1453  
 EDWARDS, JOSEPH C, CHARLES, CECIL M AND MACBRIDE, CYRIL M Evaluation of the use of anterior pituitary extract in the treatment of pituitary dwarfism 1615  
 ELLIOTT, K A C (See RABINOVITCH, ELLIOTT AND McEACHERN) 294  
 EPSTEIN, JEROME H (See GOLDSTEIN, EPSTEIN AND ROE) 1047  
 EISEN, M (See STOKSTAD, PAGE, HERCE, FRANKLIN, JUKES, HEINLE, EPSTEIN AND WELCH) 860  
 ESSEX, HIRAM E (See BERRY, BALDES, ESSEX AND WAKIM) 101  
 EVANDER, LEONARD C AND DOYLE, WILLIAM M Trichostrongylus infection in human beings need of differentiation from hookworm 969  
 EVANS, SILAS M AND ZEIT, WALTER The physical mechanism of fibrotic reactions 1454
- F
- FABRIZIO, ANGELINA (See HAMBURGER, BERMAN AND FABRIZIO) 1460  
 FARBER, EUGENE M (See HINES AND FARBER) 1486  
 FARBER, SEYMOUR M, EAGLE, HENRY R AND ANDERSON, HAMILTON H AND GORMAN, RALPH D Topical application of subtilin to tuberculous lesions in man 799  
 FARNSWORTH, EDITH B AND KRAKUSIN, JACOB S (With the technical assistance of Hughes Lora Belle) Electrolyte partition in patients with edema of various origins Qualitative and quantitative definition of cations and anions in cardiac decompensation 1334  
 — AND — Electrolyte partition in patients with edema of various origins Qualitative and quantitative definition of cations and anions in hepatic cirrhosis 1545  
 FAWCETT, MARGARETTE L (See HATTERSLEY AND FAWCETT) 1177  
 FAY, IRENE (See CARTWRIGHT, FAY, TATTING AND WINTROBE) 397  
 FEIGELSON, PHILIP (See MANWELL AND FEIGELSON) 777  
 FLIGIN, E V AND WEISMAN, S A Dieu marol and quimidine in the ambulatory treatment of chronic auricular fibrillation 1492  
 FELDMAN, M JR (See CALLEBAUT, FELDMAN, ROBBARD AND KATZ), 1495  
 FELL, MARY B (See HUNT AND FELL), 886  
 FERAYONI, RICHARD R (See MORLOCK, FERAYONI AND CODE) 1622  
 FERRIS, EUGENE B (See BRUST, ASSALI AND FERRIS) 1466  
 FLETCHER, H H (See TAYLOR, CORCORAN, FLETCHER AND PAGE), 1469  
 FIDLAR, E AND JACQUES, L B The effect of commercial heparin on the platelet count 1410  
 FINCH, CLEMENT A (See RATH AND FINCH) 81  
 FINDLEY, JOHN W, JR, KIRSNER, JOSEPH B AND PALMER, WALTER LINCOLN The incidence of symptoms and the gastric secretory response to histamine in patients with and without chronic gastritis, 1624  
 FINEBERG, SAMUEL AND BERNSTEIN, THEODORE B Histamine antagonists A new antihistaminic drug 2 [a (2 dimethylaminoethoxy) a methyl benzyl] pyridine succinate (decapryl succinate) experimental and clinical results 319  
 FINLAND, MAXWELL (See BARNES, MORGAN AND FINLAND) 309  
 — (See MORGAN, BARNES AND FINLAND) 1212  
 — BARNES, MILDRED W, MEADS, MANSON AND ORY, EDWIN M Serologic studies of influenza made in Boston during the winter of 1945 1946 1  
 — ORY, EDWIN M, MEADS, MANSON, AND BARNES, MILDRED W Influenza and pneumonia. Serologic studies during

- and after an outbreak of influenza B, 32
- FISCHER, JACK W (See CALLEN AND FISCHER), 1500
- FISCHER, R G A device to facilitate the cleaning of cages containing cotton rats, 519
- FISCHMAN, ANDOR (See FISCHMANN AND FISCHMAN), 772
- FISCHMANN, E J, AND FISCHMAN, ANDOR The thiocyanate content of saliva in normal and hypertensive subjects before and after ingestion of the drug, 772
- FISHMAN, WILLIAM H (See ODELL AND FISHMAN), 1619
- FITCH, HOWARD M (See MEYER, SAWITSKY, RITZ, AND FITCH), 189
- (See SAWITSKY, FITCH, AND MEYER), 203
- FLINN, JOHN HASLOP The effect of digitalis on the coagulability of blood, 1653
- FORREST, R A (See GRIMELLI, CHERTACK, RHETTA, KENDRICK, AND FORREST), 1617
- FRANCE, WESLEY G (See BROWN, READ, WISEMAN, AND FRANCE), 1523
- FRANKLIN, A L (See STOKSTAD, PAGE, PURCE, FRANKLIN, JUKES, HEINLE, EPSTEIN, AND WELCH), 860
- FRANKLIN, MURRAY, POPPER, HANS, STEIGMANN, FREDERICK, AND KOZOL, DONALD D Relation between structural and functional alterations of the liver 435
- FRAZIER, LAURENCE E (See BENDITT, HUMPHREYS, WISLER, STEFFEE, FRAZIER, AND CANNON), 257
- FREEMAN, SMITH (See MENG AND FREEMAN), 659
- FREIER, ESTHER F, AND LARSON, EVREL A A microadapter for the Evelyn microcolorimeter 1594
- FILMES, I ARTHUR (See DONOHUE AND FREMES), 526
- FREYBERG, R H (See GILLMOR AND FREYBERG), 1024
- FRIEDLAENDER, ALEX S (See FRIEDLAENDER AND FRIEDLAENDER), 865
- FRIEDLAENDER, SIDNEY, AND FRIEDLAENDER, ALEX S Experimental and clinical studies of neohexamine, a new antihistamine agent, 865
- FRTZ, HERBERT (See WADE, NEUDORFF, FRITZ, AND KARL), 1123
- FUTCH, E D (See MAY, BENNETT, LANE, FUTCH, SCHOOMFR, AND GREGORY), 1494
- AND GREGORY, RAYMOND Cholesterol tolerance tests in normal, diabetic, and hypertensive patients, 1618
- G
- GABUZDA, G J (See FORHAUS, SCUDAMORE, GABUZDA, MOLEY, MALONEY, AND KAPK), 1475
- GAIL, E A (See COGSWELL, CLEVELAND, GALL, WEISBPOT, AND SCHIFF), 1627
- GARDNER, FRANK H (See WAGLEY, SHEN, GARDNER, AND CASTLE), 1197
- GAPVER, W P Castor bean sensitivity, 1613
- GAPVLY, J L (See PETEFMAN AND GARVEY), 1620
- GASTON, EVELYN (See JACOBSON, MARKS, GASTON, ALLEN, AND BLOCK), 1566
- GEIB, NAOMI C (See BLOCK, GEIB, AND ROBINSON), 1381
- GEPHARDT, MAURICE (See ABRAMSON, COIF, KEETON, GEPHARDT, DYNIEWICZ, PRESLEY, AND LAVERS), 1474
- GEYER, ROBERT P (See MANN, GEYER, WATKIN, SMYTHE, ZANCHECK, AND STARE), 1503
- , MANN, GEORGE V, AND STARE, FREDRICK J Parenteral nutrition IV Improved techniques for the preparation of fat emulsions for intravenous nutrition, 153
- , —, AND — Parenteral nutrition VI Fat emulsions for intravenous nutrition the turbidimetric determination of infused fat in blood after intravenous administration of fat emulsions, 175
- , —, YOUNG, JOHN, KINNEY, THOMAS D, AND STARE, FREDRICK J Parenteral nutrition V Studies on soy bean phosphatides as emulsifiers for intravenous fat emulsions, 163
- GIFREE, JOSEPH W (See KNAPP, GIFREE, AND JACKSON), 1490
- GILBERT, N C, AND NIEFSKI, LESTER The effect of the anticoagulant drugs upon the coronary flow, 1455
- GILLMOR, C S, AND FREYBERG, R H The effect of 2,3 dithiopropanol (BAL) on gold toxicity in rats, 1024
- GLASER, KURT, PONCHER, HENRY G, AND LINDARZI, LOUIS R The cellular composition of the bone marrow in normal infants and children, 1639
- GLAUBIGER, ALBERT The purification of diptheria and tetanus antitoxin by the use of pepsin, 757
- GLEN, WILLIAM W L (See CRESSON AND GLEN), 1597
- GNESH, GLADYS M (See MALTANER AND GNESH), 383
- GOLD, DAVID (See BRAUDE AND GOLD), 1499
- GOLDBLOOM, A ALLEN (See LIEBERSON AND GOLDBLOOM), 118
- GOLDEN, WALTER R C, AND SANVELY, JOHN C A method for the quantitative determination of bilirubin in urine, 890
- GOIDMAN, MELVIN L (See SCHROEDER AND GOLDMAN), 1470
- GOLDSTEIN, NORMAN P, EPSTEIN, JEFOME H AND ROE, JOSEPH H Studies of pancreatic function IV A simplified method for the determination of serum lipase, using aqueous tributyrin as substrate, with one hundred normal values by this method, 1047

- GOLDZIEHEL, JOSEPH W A new colorimetric method for the determination of pregnandiol, 251
- GOLLS ORVILLE J Acquired resistance of psittacosis virus to sulfadiazine and effects of chemical antagonists on sulfonamide activity, 1241
- GOODRICH, BEN E BEIRMAN VIVIAN G AND HARTMAN FRANK W Studies on cardiorespiratory function with the oxyhemograph 1454
- GOOTNICK ABRAHAM (See BALKIN AND GOOTNICK) 97
- GORDON, EYE B (See WIENER AND GORDON) 181
- GORMAN RALPH D (See FARBER EAGLE ANDERSON AND GORMAN) 799
- GRANT, HARRY L (See SUTTON WENDEL AND GRANT), 1502
- (See SUTTON, WENDEL GRANT AND WARNICK), 1501
- GRAY, S H (See SHUTZ BERGMANN AND GRAY), 1558
- GRAYZEL, DAVID M (See LOEWE HIRSCH GRAYZEL AND KASHDAN) 721
- GREEN, IRDA ALDEN (See PAGE RAPPORT AND GREEN) 1606
- GREEN, D V Mechanisms of desoxycorticosterone action I. Relation of fluid intake to blood pressure 553
- JOHNSON A DEAN LOBB ALLEN AND CUSICK GEORGE The effects of adrenalin in normal and hypertensive patients in relation to the mechanism of sustained pressure elevations 332
- GREEN, ROBERT S (See MCGUIRE GREEN COFFET NOERTKER IGLAUER HAUENSTEIN AND BRAUNSTEIN) 149
- IGLAUER ARNOLD AND MCGUIRE JOHNSON Alterations of radial or brachial intra arterial blood pressure and of the electrocardiogram induced by tilting 931
- , — and — Intra arterial blood pressure curves during tilting 1483
- GREEN, JAMES A (See HARRISON GREENE AND MORSE) 1608
- GRIFENWALT, THOR J AND GRUENINGER ANTHONY J Clinical observations with methyl blue ( $\beta$  chloroethyl) amine hydrochloride 1648
- GREGORY RAYMOND (See Futch and Gregory) 1618
- (See MAY BENNETT LANE FUTCH SCHODDER AND GREGORY) 1494
- GRIMELLI LUKE J (See CHERTACK GRIMELLI RHETTA AND KEETON) 1616
- CHERTACK M M RHETTA H L KEEDRICK A B AND FORREST R A Effect of hyperglycemia on the clearance of inulin and para aminohip puric acid 1617
- GRINDLAY JOHN H (See BOLLMAN CAIN AND GRINDLAY) 1349
- GRINSTEIN MOISES KAMEN M D AND MOORE CARL A Studies on globin and porphyrin metabolism made with  $C^{14}$  and  $N^{15}$  1478
- GROOM DALE WOOD EARL H, AND ODEL HOWARD M The recording of oxygen saturation of hemoglobin by a whole blood oximeter and its application to clinical investigation 1637
- GROSSMAN, BURTON J (See ALLEN JACOBSON AND GROSSMAN) 1480
- GRIENINGER ANTHONY J (See GREENWALT AND GRUENINGER) 1648
- GUGLE LLOYD J (See CHAPMAN AND GUGLE) 1483
- GUSTAFSON WESLEY A (See MEREDITH HICK AND GUSTAFSON) 1493
- GUTERMAN HENRY S, AND SCHROEDER, MAD ELINE S A simplified technique for the quantitative colorimetric estimation of pregnandiol in urine 356
- GUYTON ARTHUR C AND MILLER, GEORGE L JR Apparatus for producing quantitative ischemia in the limbs of animals 1450

## H

- HAGEDORN A B (See STICKNEY HAGEDORN MILLS AND COOPER) 1481
- HALLMAN PAUL O AND CHIFFELLE THOMAS R Ethylene glycol poisoning A clinical and pathologic study of three cases 373
- HAGEN IANL STICKNEY AND WATSON CECIL JAMES Idiopathic (familial) hypoprothrombinemia 542
- HALL B E AND CAMPBELL D C Effect of vitamin  $B_{12}$  on the hematopoietic and nervous systems in Addisonian pernicious anemia 1646
- HALL JAMES W (See SMITH AND HALL), 998
- HALL LOIS (See CHOW, HALL, DUFFY AND ALPERT) 1440
- HALLIDAY SIBELLA (See HYPNED CUNNINGHAM HALLIDAY VESSEY LUDA CLARK HINE COSCROVE AND SUBBAROW) 216
- HALPERN B (See DOLKART HALPERN KUTZ LESHI WOLNIACK AND DEX) 1608
- HAMBURGER, MORTON BERMAN, JEROME R, AND PABIZIO ANGELINA The development of streptomycin resistant bacteria in the stools of patients treated for tuberculosis 1460
- HAMILTON HENRY E SHEETS RAYMOND F AND DEGOWIN ELMER L A method of frequent estimations of circulating plasma volume with erythrocyte counts adaptable to long continued observations 1650
- HAMMOND ROBERT L AND WELCHER MERRILL L Porphobilinogen tests on a thousand miscellaneous patients in a search for false positive reaction 1254
- HANNAN JOHN AND WEAVER R H Quick microtechniques for the identification of cultures II. Fermentations 1338
- HARDGROVE MAURICE AND MENDELSON DANIEL J Dibenamine 1496
- HAJDT LEO L (See SCHLESINGER HAJDT AND STEIGMANN) 1626

- HARNED, B K, CUNNINGHAM, RAYMOND W, HALLIDAY, SYBELLA, VESSEY, R E, YUDA, N N, CLARK, MARY C, HINE, CAROLYN H, COSGROVE, RACHEL, AND SUBBAROW, Y Studies on the chemotherapy of filariasis VI Some pharmacodynamic properties of 1 diethylcarbonyl 4 methylpiperazine hydrochloride, hetrazan, 216
- HARRISON, PRESTON E, GREENE, JAMES A, AND MORSE, ROBERT A Haverhill fever (*Haverhillia multiformis* bacteremia) the bacteriologic diagnosis, 1608
- HARTMAN, FRANK W (See GOODRICH, BEHRMANN, AND HARTMAN), 1454
- HASS, GEORGE M (See SCHICK AND HASS), 1612
- (See TAYLOR, HASS, AND MALONEY), 1462
- (See TAYLOR, SLAUGHTER, PRESTON, CRUMRINE, AND HASS), 1645
- HATTERSLEY, PAUL G, AND FAWCETT, MARGARET L The use of preserved erythrocytes for the detection and identification of Rh antibodies, 1177
- HAUENSTEIN, VIRGIL (See MCGUIRE, GREEN, COURTER, NOERTKEP, IGLAUER, HAUENSTEIN, AND BRAUNSTEIN), 1457
- HAVEL, L (See HORLICK AND HAVEL), 1029
- HAYES, DANIEL W, WAKIL, KHALIL G, HORTON, BAYARD T, AND PETERS, GUSTAVUS A The effects of dihydroergocornine on the peripheral circulation in man, 1497
- HAYLES, ALVIN B (See SPRAGUE, HAYLES, MASON, POWER, AND BENNETT), 1472
- HEFTMANN, ERICH (See WILKERSON AND HEFTMANN), 236
- HEGSTED, D M (See WANG, LAPE, AND HEGSTED), 462
- HEINLE, R W (See STOKSTAD, PAGE, PIERCE, FRANKLIN, JUKES, HEINLE, EPSTEIN, AND WELCH), 860
- , WELCH, ARNOLD D, AND PRITCHARD, JACK A Essentiality of both the antipernicious anemia factor of liver and pteroylglutamic acid for hematopoiesis in swine, 1647
- HELLERSTEIN, HERMAN K, AND LIEBOW, IRVING M Factors influencing the T wave of the electrocardiogram, an experimental study employing intracavitary and epicardial leads I Effects of heating and cooling of subepicardial and subendocardial lamina, 1498
- HELMER, O M, SHIPLEY, R E, PEIRCE, J D, AND KOHLSTAEDT, K G Antibodies against renin and "sustained pressor principle" produced by injections of kidney extracts, 1484
- HEMINGWAY, ALLAN, BERNAT, LEO ALLEN, AND MASCHMEYER, JOSEPH Determination of volatile reducing substances (alcohol or ether) in blood and gases using barium diphenylamine sulfonate as an indicator for chromic acid titration, 126
- HENRY, JANE (See HENRY, KIRKWOOD, BERKMAN, HOUSEWRIGHT, AND HENRY), 241
- HENRY, RICHARD J, KIRKWOOD, CAROL F, BERKMAN, SAM, HOUSEWRIGHT, RILEY D, AND HENRY, JANE A colorimetric method for the determination of microquantities of ethanol in blood and other biologic fluids, 241
- HERRELL, WALLACE E (See NICHOLS AND HERRELL), 521
- HERSHEY, J IVAN (See STRUMIA AND HERSHEY), 1311
- HICK, FORD K (See MEREDITH, HICK, AND GUSTAFSON), 1493
- HINE, CAROLYN H (See HARNED, CUNNINGHAM, HALLIDAY, VESSEY, YUDA, CLARK, HINE, COSGROVE, AND SUBBAROW), 216
- HINES, EDGAR, A, JR, AND FABER, EUGENE M The arterioles of the skin in essential hypertension, 1486
- HIRATZKA, TOMIHARU (See MIERS, KLEIN, AND HIRATZKA), 1456
- HIRSCH, EDWARD (See LOEWE, HIRSCH, GRAYZEL, AND KASHDAN), 721
- HIRSCHBERG, ERICH (See PARSONNET, BERNSTEIN, KLOSK, HIRSCHBERG, RUBIN, AND PIRK), 602
- HIRSCHBOFCK, JOHN S The effect of operation and illness on clot retraction description of a new method, 347
- HURSH, HAROLD L (See ROBINSON, HURSH, MILLOFF, AND DOWLING), 1232
- , WELCH, HENRY, MILLOFF, BERNARD, AND KATZ, SOL Administration of penicillin and streptomycin by means of the hypodermic apparatus (jet injection), absorption, toxicity, and stability, 805
- HOFFMAN, WILLIAM S (See MARSHALL AND HOFFMAN), 1468
- AND MARSHALL, DANIEL Management and clinical course of lower nephron nephrosis, 1606
- AND NOBE, CATHERINE Renal excretion of salicylate during treatment of acute rheumatic fever, 1636
- , KOZOLL, DONALD D, MOR, W T, MEYER, KARL A, AND POPPER, HANS The determination of the nitrogen balance index of a new lyophilized amino acid preparation in protein deficient patients, 280
- HOLT, GORDON (See JACOBSON, LEVIN, AND HOLT), 1641
- HOLT, REBECCA A, AND NEWTON, HAZEL The occurrence of *Salmonella blegdam* in Louisiana, 1155
- HOMBURGER, F (See CHOW, HOMBURGER, DEBIASE, AND PETERMAN), 1052
- HOEBLER, S W, MANNING, J T, MCCLELLAN, S G, RENVERT, HENRY, JR, AND PEET, MAX M Effects of splancnectomy on blood pressure and on cardiac and renal function, 1485

HORAN THOMAS N (See BERMAN AXELIOD JACOBSON HORAN VONDERHEIDE AND SHAPIR), 1628

HORECKER B L Crystalline hemin solutions as permanent standards for hemoglobin estimation 781

HORICK, L AND HAVEL, L The effect of feeding propylthiouracil and cholesterol on the blood cholesterol and arterial intima in the rat 1029

— AND KATZ L N The effect of diethyl stilbestrol on blood lipids and the development of atherosclerosis in chickens on a normal and low fat diet 733

HORNBER, JOHN L Premature calcification of the costal cartilages its frequent association with psychoneuroses and possible endocrine imbalance 16-1

HORTON, BAYARD T (See HAYES WARREN HORTON AND PETERS) 1497

— (See MCELIN AND HORTON) 1610

— AND WAGENER, HARRY P Retrobulbar neuritis treatment with histamine 1611

HOSMER HERMAN A (See LINES DON AND HOSMER) 1002

HOU TUNG CHANG (See CHANG AND CHANG) 1447

HOUSEWRIGHT RILEY D (See HENRY KIRK WOOD BRYAN HOUSEWRIGHT AND HENRY) 241

HOWITT, BEATRICE AND BARNETT VOHAMMIF H Use of penicillin and streptomycin in the isolation of polyomyelitis virus from fecal specimens 1402

HUGHES LORA BELLE (See FARNSWORTH AND KRAKUSIN) 1534 1545

HUMPHREYS ELEANOR M (See BENDITT HUMPHREYS WISSLER STEFFEE FLAZIER AND CANNON) 257

HUNT ANDREW D JR, AND FELL MARY B A micromethod for the determination of serum streptolysin levels 886

## I

IGLAUER ARNOLD (See GREEN IGLAUER AND MCGUIRE) 951 1483

— (See MCGUIRE GREEN COURTER NOFTKER IGLAUER HADENSTEIN AND BRAUNSTEIN) 1457

## J

JACKSON ROBERT L (See KNAPP GIBBEL AND JACKSON) 1490

JACOBS, WILLIAM (See DAVIS SEGATOFF AND JACOBS) 1483

JACOBSON LEON O (See ALLEN JACOBSON AND GROSSMAN) 1480

— (See BLOCK JACOBSON, NEAL AND SMITH) 1649

— MARKS EDNA K GASTON EVELYN ALLEY J GARROTT AND BLOCK MATTHEW H The effect of nitrogen mustard and X irradiation on blood coagulation, 1566

JACOBSON SAMUEL D (See BERMAN AXELIOD JACOBSON HORAN, VONDERHEIDE AND SHAPIR) 1628

JACOBSON W LEVIN WILLIAM C AND HOLT GORDON Observations on the treatment of acute leucemias with analogues of folic acid 1641

JAMES GEORGE W III RIBLET LILLIAN A ROBINSON JOSEPH C JOHNSON ROBERT E AND KARK ROBERT M Studies on prolonged supplicative infection in man, 1607

JANOWITZ HENRY D SCHNEIERSON S STANLEY SUSSMAN MARCO L AND KING, FREDERICK H The role of high blood penicillin levels achieved with caronamide in penetrating the blood brain barrier 933

JAKES L B (See FIDLAR AND JAQUES) 1410

JACKSON RONALD N (See SINGER, ROBIN KING AND JEFFERSON) 975

JOHNSON A DEAN (See GREEN JOHNSON, LOBB AND CLSICK), 332

JOHNSON MARY C (See BUKANTZ DAMMIL WILSON AND JOHNSON) 1463

JOHNSON ROBERT E (See JAMES RIBLET ROBINSON JOHNSON AND KARK) 160

JONES T H (See STORSTAD PAGE PIERCE, FRANKLIN JAMES HEINLE EPSTEIN AND WELCH) 860

## K

KAMLER PAUL W (See KIMBALL AND KAMLER) 154

KAHN REUBEN L (See McDERMOTT AND KAHN) 1-24

— AND McDERMOTT ELIZABETH B Studies on cardiolipin antigen IV Variations in sensitivity of different lots of purified lecithin 1220

KAMEN M D (See GRUNSTEIN KAMEN AND MOORE) 1418

KARK ROBERT M (See JAMES RIBLET, ROBINSON JOHNSON AND KARK) 1607

— (See VOHRHAUS SCUDAMORE GABUZDA MOOREY MALONEY AND KARK) 1470

KARL MICHAEL (See WADE NELDORFF FRITZ AND KARL) 11-3

KASHDAN FLORENCE (See LOEWE HIRSCH GRAYZEL AND KASHDAN), 7-1

KATZ L N (See CALLEBAUT FELDMAN ROBBARD AND KATZ), 1495

— (See HORLICK AND KATZ) 733

— (See MILLS CISNEROS AND KATZ) 1485

KATZ SOL (See HIRSH WELCH MILLOFF AND KATZ) 803

KEATING F R (See CHILDS KEATING WILLIAMS AND POWER) 1610

KEATING JOHN U AND CODE CHARLES F The anesthetic and antihistaminic action of a series of antihistaminic drugs in human skin 1609

- KEETON, ROBERT W (See ABFAMSON, COLE, KELTON, GEPHARDT, DYNIEWICZ, PRESLEY, AND LAVERS), 1474  
 — (See ALLEN AND KEETON), 1500  
 — (See CHERTACK, GRIMELLI, RHETTA, AND KEETON), 1616
- KENDRICK, A B (See GRIMELLI, CHERTACK, RHETTA, KENDRICK, AND FORREST), 1617
- KENT, JOHN F, CULWELL, WILLIAM B, COATNEY, G ROBERT, AND COOPER, W CLARK False positive tests for syphilis a further study of their incidence in sporozoite induced vivax malaria, 747
- KIBRICK, ANDRE C, AND CLEMENTS, ALFRED B A comparative study of the serum albumin globulin ratio, the cephalin cholesterol flocculation, and the thymol turbidity tests for liver function, 662
- KIMBALL, ANNE C, AND KABLER, PAUL W The effect of chlorine on complement fixation, 254
- KING, FREDERICK H (See JANOWITZ, SCHNEIERSON, SUSSMAN, AND KING), 933
- KING, JOSEPH C (See SINGER, KING, AND ROBIN), 1068  
 — (See SINGER, ROBIN, KING, AND JEFFERSON), 975
- KINNEY, THOMAS D (See COLLINS, KRAFT, KINNEY, DAVIDSON, YOUNG, AND STARE), 143  
 — (See GEYER, MANN, YOUNG, KINNEY, AND STARE), 163
- KIRKWOOD, CAROL F (See HENRY, KIRKWOOD, BERKMAN, HOUSEWRIGHT, AND HENRY), 241
- KIRSNER, JOSEPH B (See FINDLEY, KIRSNER, AND PALMER), 1624  
 — (See LEVIN, KIRSNER, AND PALMER), 1623  
 — (See RICKETTS, KIRSNER, AND PALMER), 1475
- KITZBERGER, DORIS M (See THOMA AND KITZBERGER), 1189
- KLAPMAN, MARTIN J (See PETERS, LINDERT, KLAPMAN, AND MENDELSON), 1619
- KLEIN, HOWARD A (See MYEPS, KLEIN, AND HIRATZKA), 1456
- KLING, DAVID H Failure of antireticular cytotoxic serum in arthritis, 1289
- KLOSK, EMANUEL (See PARSONNET, BERNSTEIN, KLOSK, HIRSCHBERG, RUBIN, AND PIK), 602
- KNAPP, ELIZABETH, GIFFEE, JOSEPH W, AND JACKSON, ROBERT L Plasma protein studies in children with rheumatic fever, 1490
- KNIBB, SHIRLEY A (See CAMPBELL AND SASLAW), 1207
- KNIGHT, ALVA A, AND MILLER, JEANNE Studies of the influence of oxyquinoline drugs on growth of *Endamoeba histolytica* as measured in blood iodine levels of man, 1632
- KOHLSTAEDT, K G (See HELMER, SHIPLEY, PEIRCE, AND KOHLSTAEDT), 1484
- KORNS, ROBERT F, AND TRUSSELL, RAY E Treatment of the typhoid carrier state. Trial of two chemotherapeutic procedures, 1150
- KOZOLL, DONALD D (See FRANKLIN, POPPER, STEIGMANN, AND KOZOLL), 435  
 — (See HOFFMAN, KOZOLL, MOK, MEYER, AND POPPER), 280  
 — AND MACNEAL, GORDON Comparative nitrogen balance studies of oral protein derivatives and natural protein in protein deficient patient, 1634
- KRAEMER, MANFRED (See SIEGEL AND KRAEMER), 618
- KRAFT, LISBETH M (See COLLINS, KRAFT, KINNEY, DAVIDSON, YOUNG, AND STARE), 143
- KRAKUSIN, JACOB S (See FARNSWORTH AND KRAKUSIN), 1534, 1545
- KRAUSHAR, OTTO F (See BROWN, BRADBURY, AND KRAUSHAR), 1465  
 — AND BRADBURY, JAMES T A modified technique for the examination of body fluids, 1195
- KRUGER, H E (See BERGMAN, KRUGER, AND PRINZMETAL), 506  
 — (See PRINZMETAL, KRUGER, AND BERGMAN), 497  
 — AND PRINZMETAL, MYRON The mechanism of bacteria induced shock resulting from crushing of muscle in dogs, 502
- KUSHNER, S (See MCKENZIE, MALONE, KUSHNER, OLESON, AND SUBBAROW), 1249
- KUTZ, A L (See DOLKART, HALPERN, KUTZ, LESH, WOLNIAK, AND DEY), 1608
- L
- LANDOWNE, MILTON, AND THOMPSON, WALTER S Failure of antihistaminic drugs to reduce reactive hyperemia in man, 1609
- , —, AND RUBY, BARBARA The minimal sodium diet A controlled study of its effect upon the blood pressure of ambulatory hypertensive subjects, 1482
- LANE, A L (See MAY, BENNETT, LANE, FUTCH, SCHOOVER, AND GREGORY), 1494
- LAPI, A (See WANG, LAPI, AND HEGSTED), 462
- LAPCOM, RODNEY C, JR, AND CARTER, GEORGE H Erythrocytes in urinary sediment identification and normal limits, with a note on the nature of granular casts, 875
- LARSON, EVRELL A (See FREIER AND LARSON), 1594
- LASNER, JACK (See DE TARATS AND LASNER), 1487
- LAVERS, GEORGE (See ABRAMSON, COLE, KEETON, GEPHARDT, DYNIEWICZ, PRESLEY, AND LAVERS), 1474
- LAWRENCE, JOHN S, VALENTINE, WILLIAM S N, AND ADAMS, WILLIAM S Thrombopenic purpura, the failure of direct blood transfusion to raise the platelet level, 1077

- LAYNE, J A (See SCHEMM AND LAYNE), 1467
- LAZAROW, ARNOLD Screw syringe, 1603
- LEARD, SAMUEL E (See CURRY AND LEARD) 585
- LECH, RACHEL S, AND WOODFORD NORMA A simple bedside method for the estimation of blood sugar 644
- LEIN, MARILAN (See ALBANESE AND LEIN), 246
- LEROY, G V, AND NALEFSKI L A Dicumarol in experimental myocardial infarction, 961
- , — AND CHRISTY, HAROLD W The effect of certain sympatholytic agents on the coronary blood flow of the dog 1496
- LESH, J B (See DOLKART, HALPERN KUTZ LESH, WOLNIAR AND DEY) 1608
- LEVIN, ERWIN, KIRSNER, JOSEPH B AND PALMER WALTER LINCOLN A comparison of the twelve hour nocturnal gastric secretion in uncomplicated duodenal ulcer before and after healing, 1623
- LEVIN, WILLIAM C (See JACOBSON, LEVIN AND HOLT), 1641
- LEWIS, M N (See SCHWARTZ AND LEWIS) 904
- LIEBERSON, ABRAHAM AND GOLDBLOOM A ALLEN Devices for rapid recording of multiple and special electrocardiographic leads 118
- LIEBOW, IRVING M (See HELLERSTEIN AND LIEBOW) 1498
- LIMARZI, LOUIS R (See GLASER, PONCHER AND LIMARZI) 1639
- AND PAUL JEROME T Sternal marrow studies in Hodgkin's disease 1640
- LINDERT, M C F (See PETERS LINDERT KRAIMAN AND MENDELSON) 1619
- AND CORRELL HOWARD L The appearance of normoblasts in the peripheral blood in patients with pulmonary embolism 1502
- LITTLE, J MAXWELL (See WALLACE LITTLE AND BOBB) 845
- LITTLE, PAUL A OLESON J J AND SUBBAROW Y The effect of nutrition on the tumor response in Rous chicken sarcoma 1139
- SAMPATH, ANGUS AND SUBBAROW Y The use of antagonists of pteroyl glutamic acid in controlling Rous chicken sarcoma 1144
- LOBB, ALLEY (See GILLEY, JOHANSON LOBB AND CUSICK) 332
- LOEWY, LEO HIRSCH EDWARD GRAYZEL DAVID M. AND KASIDAN FLORENCE Experimental study of the comparative action of heparin and dicumarol on the *in vivo* clot 721
- LOOSI, CLAYTON G SCHOENBERGER JAMES AND BARNETT GWENDOLYN Results of vaccination against influenza during the epidemic of 1947 789
- LOUIS, LAWRENCE H (See CONE LOUIS AND WHEELER), 651
- (See ROBINSON CONN, BLOCK AND LOUIS), 1473
- MC
- MACBRYDE, CYRIL M (See EDWARDS, CHARLES, AND MACBRYDE) 1615
- MACNEAL GORDON (See KOZOLL AND MACNEAL) 1634
- MCBURNEY, RALPH, AND CASOV, LOUISE R Use of splenic infusion as a base for growing certain pathogenic microorganisms, 1037
- MCLELLAN, S G (See HOOBLER, MANNING, MCCLELLAN RENFERT, AND PEET), 1485
- MCDERMOTT ELIZABETH B (See KAHN AND MCDERMOTT) 1220
- AND KAHN REUBEN L Studies on cardiolipin antigen V Standardization of antigen for the Kahn test 1224
- MCACHERN, DONALD (See RABINOVITCH ELLIOTT AND MCEACHERN), 294
- MCEIN, THOMAS W, AND HORTON, BAYARD T Clinical observations on histamine administration during pregnancy, 1610
- MCGWACK THOMAS H WEISSEBERG, JONAS AND BOYD LIN J A comparison of the toxic manifestations produced by beta dimethylaminoethyl benzhydrol ether hydrochloride (benzhydrol) and tripeleannamine (pyribenzamine) 595
- MCGUIRE JOHNSON (See GREFF, IGLAUER AND MCGUIRE) 951 1483
- GREEN ROBERT COUTTER, SANFORD R NOETTKER JEAN, IGLAUER ARNOLD HALENSTEIN VIRGIL AND BRAUNSTEIN JOHN Bedpan deaths 1457
- MCKENZIE D MAYONE L KUSHNER, S OLESON J J AND SUBBAROW Y The effect of nicotinic acid amide on experimental tuberculosis of white mice 1249
- M
- MACHLE WILLARD Berylliosis observations and report of clinical study of seventy cases of chronic disease 1613
- MADISON, FREDERICK W (See BELFUS AND MADISON) 1651
- MAJUMDER D N AND WINTROBE M M Anemia of infection IX Influence of adrenalectomy and of adrenal cortical hormone on hypoferremia and other blood changes associated with injection of turpentine, 332
- MALONE L (See MCKENZIE MALONE, KUSHNER OLESON AND SUBBAROW), 1249
- MALONEY JOHN E (See TAYLOR, HASS AND MALONEY) 1462
- MALONEY M A (See VORHAUS, SCUDAMORE GABUZDA MOFEY MALONEY AND KARK) 1475
- MALTNER ELIZABETH AND GNESH GLADYS M A method for the determination of titers between 10 and 100 in the quantitative complement fixation test for syphilis 393

- MANDEL, EMANUEL E, AND PAPIIS, DELMO A The routine use of the serum flocculation reaction with Haven's solution, 1629
- AND THAYFF, JAMES D Rectal absorption of penicillin, 135
- MANN, GEORGE V (See GEYER, MANN, AND STARE), 153, 175
- (See GEYER, MANN, YOUNG, KINNEY, AND STARE), 163
- AND STARE, FREDRICK J Nutrition and experimental diabetes I The diuretic response of weanling rats to intravenous doses of alloxan, 1161
- , GEYER, ROBERT P, WATKIN, DONALD M, SMYTHE, RICHARD L, ZAMCHECK, NORMAN, AND STARE, FREDRICK J Parenteral nutrition VII Metabolic studies on puppies infused with fat emulsions, 1503
- MANNING, J T (See HOOBLER, MANNING, MCCLELLAN, RENEFT, AND PEFT), 1485
- MARWELL, REGINALD D, AND FEIGELSON, PHILIP A modified method of preparing the JSB strain, 777
- MARKS, EDNA K (See JACOBSON, MARKS, GASTON, ALLEN, AND BLOCK), 1566
- MARSHALL, DANIEL (See HOFFMAN AND MARSHALL), 1606
- AND HOFFMAN, WILLIAM S The nature of the altered renal function in lower nephron nephrosis, 1468
- MASCHMEYER, JOSEPH (See HEMINGWAY, BERNAT, AND MASCHMEYER), 126
- MASON, H L (See SPRAGUE, HAYLES, MASON, POWER, AND BENNETT), 1472
- MASSIE, EDWARD, AND WIEGAND, HERBERT C Pulpitation, 1487
- MASSON, G, COPCORAN, A C, AND PACE, IRVINE H Dietary and hormonal influences in experimental anuria, 1439
- MAY, LAWRENCE G, BENNETT, ALENE, LANE, A L, FUTCH, E D, SCHOOVER, MARY LYNN, AND GREGORY, RAYMOND The effect of apprehension caused by the technical procedures on cardiac output, 1494
- MEADS, MYNOR (See FINLAND, BARNES, MEADS, AND OPI), 15
- (See FINLAND, ORY, MEADS, AND BARNES), 32
- MENDELSON, DANIEL J (See HARDGROVE AND MENDELSON), 1496
- (See PETERS, LINDEPT, KLAPMAN, AND MENDELSON), 1619
- MENDEZ, RAFAEL (See MOE AND MENDEZ), 1491
- MENG, H C, AND FREEMAN, SMITH Experimental studies on the intravenous injection of a fat emulsion into dogs, 689
- MEREDITH, PAUL A, HICK, FORD K, AND GUSTAFSON, WESLEY A Paroxysmal tachycardia, low nodal in origin, benefited by cardiac sympathectomy, 1493
- MERLEY, ROBERT W, AND BUEDING, ERNEST Systematic qualitative analysis of biologic materials for common steam volatile organic poisons, 371
- METCOFF, JACK, DARLING, DOROTHY B, SCANLON, MARGARET H, AND STARE, FREDRICK J Nutritional status and infection response I Electrophoretic, circulating plasma protein, hematologic, hematopoietic and immunologic responses to *Salmonella typhimurium* (*Bacillus aertrycke*) infection in the protein deficient rat, 47
- MEYER, JACOB (See SWARTS, BERNSTEIN, RAGINS, AND MEYER), 1633
- MEYER, KARL A (See HOFFMAN, KOZOL, MOK, MEYER, AND POPPER), 280
- (See STEIGMANN, ZWEIG, AND MEYER), 1627
- MEYER, LEO M (See SAWITSKY, FITCH, AND MEYER), 203
- , SAWITSKY, ARTHUR, RITZ, NORTON D, AND FITCH, HOWARD M A study of cholinesterase activity of the blood of patients with pernicious anemia, 189
- MEYERS, MURIEL C (See BETHELL, MEYERS, AND NELIGH), 1477
- MILLER, A KATHRINE (See BOGER, MILLER, TILLSON, AND SHAVER), 297
- MILLER, BERNARD J Circulation time an accurate indicator for objective measurement, 910
- MILLER, GEORGE L, JR (See GLYTON AND MILLER), 1450
- MILLER, JEANNE (See KNIGHT AND MILLER), 1632
- MILLOFF, BERNARD (See HIRSH, WELCH, MILLOFF, AND KATZ), 805
- (See ROBINSON, HIRSH, MILLOFF, AND DOWLING), 1232
- MILLS, C A, AND PORTER, MARJORIE MILLS Relation of tobacco smoking to diseases of the respiratory and circulatory systems, 1497
- MILLS, G Y, CISNEROS, F, AND KATZ, L V Observations on one hundred patients with myocardial infarction who survived up to six years, 1488
- MITIS, S D (See STICKNEY, HAGDORN, MILLS, AND COOPER), 1481
- MITHOEFER, JOHN C, AND VILFER, CARL F Glycosuria simulated by the administration of ascorbic acid its occurrence and differentiation, 1471
- MOE, GORDON K, AND MENDEZ, RAFAEL The action of several cardiac glycosides on excitability and conduction velocity in the dog heart, 1491
- MOH, JAMES F (See WITEBSKY AND MOH), 1353
- AND WITEBSKY, ERNEST Studies on Rh antibodies II The demonstration



- of a third type of Rh antibody with blocking properties 161
- AND — Studies on Rh antibodies III Analysis of a zone phenomenon in an Rh antiserum split by dialysis into four fractions 1369
- MONS, FREDERIC E. (With the technical assistance of RIAN JEAN TURNBULL) The preparation of frozen sections for use in the chemurgical technique for the microscopically controlled excision of cancer 392
- MOR, W. T. (See HOFFMAN ROZELL MOR MEYER AND POPIER) 280
- MOORE CARL A. (See GRINSTEIN KAMIN AND MOORE) 1478
- MOREY G. R. (See VORHAUS SCUDAMORE GABZDA MOREY, MALONEY AND KARK) 1475
- MORGAN, HERBERT R. (See BARNES MORGAN AND FINLAND) 309
- BARNES, MILDRED W. AND FINLAND MAX WELLS Antigenic differences among influenza A viruses including serologic response of patients 1212
- MORGAN PHILIP W. (See RIAN AND MORGAN) 1621
- MORLOCK CARL G. FERAXIONI RICHARD R. AND CODE CHARLES E. The effect of enterogastric concentrates on gastric secretion in human beings 1622
- MORSE ROBERT A. (See HARRISON GREENE AND MORSE) 1608
- MOSES CAMPBELL CRITCHFIELD FRANCES H. AND THOMAS THEODORE B. The importance of the rate of dye removal in the bromosulfalein test of liver function 148
- MUIRHEAD E. E. AND REID, ALLEN F. A resin artificial kidney 841
- MUNTZ HISCALL H. (See BECK AND MUNTZ) 1159
- MURAYAMA MAKIO A rapid method for serum calcium determination 906
- MIERS, GORDON B., KLEIN HOWARD A. AND HIRATZKA TOMIHARU Correlation of electrocardiographic and pathologic findings in infarction of the interventricular septum 1456
- N
- NALEFSKI LESTER (See GILBERT AND NALEFSKI) 1499
- (See LEROY AND NALEFSKI) 901
- (See LEROY, NALEFSKI AND CHRISTY) 1496
- NAPUT, LEO F. AND TURNBULL GEORGE C. Dangers from the use of the rice diet 1468
- NATLSON SAMUEL (See ZUCKERMAN AND NATLSON) 1322
- NEAL, WILLIAM B. JR. (See BLOCK JACOBSON NEAL AND SMITH) 1649
- NECHELES H. (See STRAUSS AND NECHELES) 612
- NELIGH, ROSAIE B. (See BETHELL MEYERS AND NELIGH) 1477

- NEUDORFF LOUIS (See WADE NEUDORFF, FITZ AND KAIL), 1123
- NEWTON HAZEL (See HOLT AND NEWTON) 1155
- NICHOLS DONALD R. AND HERRELL, WALLACE E. Penicillin in the treatment of actinomycosis 921
- NOBE, CATHERINE (See HOFFMAN AND NOBE) 1636
- NOERTKER JEAN (See MCGUIRE GREEN, COFFER NOERTKER IGLEDER, HALENSTEIN AND BRAUNSTEIN) 1457
- NORTHMAN MARTIN M. PRATT J. DENNIE AND BENOTTI JOSEPH The effect of the ligation of the pancreatic ducts and of pancreatotomy after duct ligation on serum lipase 833

## O

- ODIE HOWARD M. (See GROOM WOOD AND ODEL) 1637
- ODIE LESTER D. AND FISHMAN WILLIAM H. The beta glucuronidase activity of human endometrium 1619
- OLSON J. J. (See LITTLE, OLSON AND SIBBAROW) 1139
- (See MCKENZIE MALONE KUSHNER OLSON AND SIBBAROW) 1249
- ORDWAY NELSON L. AND SINGER RICHARD B. A simplified sedimentation rate technique with combined chart and correction nomogram, 511
- ORY EDWIN M. (See FINLAND BARNES, MEADS AND ORY) 15
- (See FINLAND ORY MEADS AND BARNES) 32

## P

- PAGE A. JR. (See STORSTAD, PAGE, PIERCE FRANKLIN JUKES HEINLE EPSTEIN AND WELCH), 800
- PAGE IRVINE H. (See CORCORAN AND PAGE), 1320
- (See MASSON CORCORAN, AND PAGE) 1489
- (See TAYLOR CORCORAN, FERTIG, AND PAGE) 1469
- RAPPOORT MAURICE M. AND GREEN ARDA ALDEN The crystallization of serotonin 1606
- PALMER WALTER LINCOLN (See FINDLEY KIRSNER AND PALMER) 1624
- (See LEVIN KIRSNER AND PALMER) 1624
- (See RICKETTS KIRSNER, AND PALMER) 1475
- PALMES EDWARD D. AND PARK CHARLES R. An improved mounting for thermocouples for the measurement of the surface temperature of the body 1044
- PANZER FRANCES (See SCHWEIGERT, B. S.) 1271
- PARIS DELMO A. (See MANDEL AND PARIS) 1620
- PARK CHARLES R. (See PALMES AND PARK) 1044

- PARMER, LEO G Blood and bone marrow concentration of atabrine and its role in aplastic anemia, 827
- PARSONNET, AARON E, BERNSTEIN, ARTHUR, KLOSK, EMANUEL, HIRSCHBERG, ERICH, RUBIN, SAUL H, AND PIRK, LEO A Evaluation of a new sedative drug (3,3 diethyl 2,4 dioxopiperidine), 602
- PAUL, JEROME T (See LIMARZI AND PAUL), 1640
- PECK, HAROLD M, AND WALLER, WILLIAM S A tidal volume recorder for anesthetized dogs, 784
- PFFFT, MAX M (See HOOBLER, MANNING, MCCLELLAN, RENFERT, AND PEET), 1485
- PEIRCE, J D (See HELMER, SHIPLEY, PEIRCE, AND KOHLSTAEDT), 1484
- PETERMAN, M G, AND GARVEY, J L Pseudohypoparathyroidism, 1620
- PETERMANN, MARY L (See CHOW, HOMBURGER, DEBIASE, AND PETERMANN), 1052
- PETERS, BRUNO J, LINDERT, M C F, KLAUFMAN, MARTIN J, AND MENDELSON, DANIEL J Diffuse pancreatic calcification with diabetes mellitus, 1619
- PETERS, EMMETT L (See BROWN AND WERNER), 325
- PETERS, GUSTAVUS A (See HAYES, WAKIM, HOPTON, AND PETERS), 1497
- PETERSON, RALPH E Hepatic dysfunction in infectious mononucleosis, with review of the literature, 1258
- PIERCE, J (See STOKSTAD, PAGE, PIERCE, FRANKLIN, JUKES, HEINLE, EPSTEIN, AND WELCH), 860
- PIERCE, MILA, AND ALT, HOWARD Treatment of acute leucemia with aminopterin (4 amino pteroylglutamic acid), 1642
- PIRK, LEO A (See PARSONNET, BERNSTEIN, KLOSK, HIRSCHBERG, RUBIN, AND PIRK), 602
- PONCHER, HENRY G (See GLASER, PONCHER, AND LIMARZI), 1639
- POPPER, HANS (See FRANKLIN, POPPER, STEIGMANN, AND KOZOLL), 435
- (See HOFFMAN, KOZOLL, MOK, MEYER, AND POPPER), 280
- (See STEIGMANN, POPPER, AND SHULMAN), 1630
- , STEIGMANN, FREDERICK, DYNIEWICZ, HATTIE, AND DUBIN, ALVIN Influence of endogenous and nutritional factors upon the plasma vitamin A alcohol, 1631
- , —, —, AND — The influence of ingestion of various lipids upon the thymol turbidity, 1630
- PORTER, MARJORIE MILLS (See MILLS AND PORTER), 1497
- POWER, M H (See CHILDS, KEATING, WILLIAMS, AND POWER), 1615
- (See SPRAGUE, HAYLES, MASON, POWER, AND BENNETT), 1472
- PRATT, T DENNIE (See NOTHMAN, PRATT, AND BENOTTI), 833
- PRESTON, FREDERICK W (See TAYLOR, SLAUGHTER, PRESTON, CRUMFINE, AND HASS), 1645
- PRIDDLE, HAROLD D (See DIECKMANN, PRIDDLE, TURNER, AND TREPTOW), 1641
- PRINZMETAL, MYRON (See BERGMAN, KRUGER, AND PRINZMETAL), 506
- (See KRUGER AND PRINZMETAL), 502
- , KRUGER, H E, AND BERGMAN, H C Effect of various lethal procedures and thermal injury on capillaries, 497
- PRITCHARD, JACK A (See HEINLE, WELCH, AND PRITCHARD), 1647
- PULASKI, EDWIN J, AND SEELEY, SAM F Further experiences with streptomycin therapy in United States Army hospitals, 1
- PUPPEL, I DARIN The medical and metabolic factors in the surgical management of hyperthyroidism, 1618

## Q

- QUICK, ARMAND J, AND STEFANINI, MARIO The concentration of the labile factor of the prothrombin complex in human, dog, and rabbit blood its significance in the differentiation of prothrombin activity, 819
- , SHANBERGE, JACOB N, AND STEFANINI, MARIO The coagulation defect in thrombocytopenic purpura, 1479
- , —, AND — The effect of heparin on platelets in vivo, 1424

## R

- RABINOVITCH, REUBEN, ELLIOTT, K A C, AND MCEACHERN, DONALD Cytochrome C intravenous administration in man, 294
- RAGINS, ALEX B (See SWARTS, BERNSTEIN, RAGINS, AND MEYER), 1633
- RANDOLPH, THERON G Acute torticollis due to food allergy, 1614
- RAPPORT, MAURICE M (See PAGE, RAPPORT, AND GREEN), 1606
- RATH, CHARLES E, AND FINCH, CLEMENT A. Sternal marrow hemosiderin A method for the determination of available iron stores in man, 81
- RAYMOND, SAMUEL, AND ZHEUTLIN, E C A Scholander micrometer burette of simple construction, 1193
- READ, JOHN T (See BROWN, READ, WISEMAN, AND FRANCE), 1523
- REID, ALLEN F (See MUIRHEAD AND REID), 841
- REINHARD, JOHN F (See SCUDI AND REINHARD), 1304
- RENFERT, HENRY, JR (See HOOBLER, MANNING, MCCLELLAN, RENFERT, AND PEET), 1485
- RHETTA, HELEN L (See CHERTACK, GRIMMELI, RHETTA, AND KEETON), 1616
- (See GRIMMELI, CHERTACK, RHETTA, KENDRICK, AND FORREST), 1617

- RHOADS PAUL S, AND SCHRAM WARREN R Bacteremia following tooth extraction prevention with penicillin and 3,4-dimethyl 5 sulfamylamide isoxazole (gantrosan), 1461
- RIBLET, LILLIAN A (See JAMES RIBLET ROBINSON, JOHNSON AND KARR), 1607
- RICHARDS R H Effect of rutin upon the re-oxidative toxicity of certain drugs 1464
- RICKETTS WILLIAM E, KITSNER JOSEPH B AND PALMER WALTER L Portal cirrhosis of the liver a clinical and functional study, 1475
- RITZ NORTON D (See MEYER, SAWITSKY RITZ AND FITCH), 189
- ROBIN, SIDNEY (See SINGER ROBIN, KING AND ROBIN), 1068
- (See SINGER ROBIN, KING AND JEFFERSON), 975
- ROBINSON JAY A HIRSH, HAROLD I MILL OFF BERNARD AND DOWLING HARRY F Procaine penicillin therapeutic efficiency and a comparative study of the absorption of suspensions in oil and in oil plus aluminum mono stearate and of an aqueous suspension containing sodium carboxymethylcellulose, 1232
- ROBINSON JOSEPH C (See JAMES RIBLET ROBINSON, JOHNSON AND KARR), 1607
- ROBINSON WILLIAM D (See BLOCK GEIB AND ROBINSON), 1381
- COHN, JEROME W BLOCK WALTER D AND LOUIS LAWRENCE H Role of the adrenal cortex in urate metabolism and in gout 1473
- RODBARD, S (See CALLEBAUT FELDMAN RODBARD AND KATZ), 1495
- ROE, JOSEPH H (See GOLDSTEIN EPSTEIN AND ROE), 1047
- ROGERS EVELYN M (See DE TAAATS AND ROGERS), 1652
- ROSENBAUM EDWARD E, AND BARKER NELSON W A test of the coagulation time of blood heparinized in vitro studies of normal subjects and of patients with intravascular thrombosis 1342
- ROSENTHAL ROBERT L AND TOBIAS CHARLES W Measurement of the electric resistance of human blood use in coagulation studies and cell volume determinations 1110
- ROTTINO ANTONIO SUCIOFF DIANA AND STERN KURT G Electrophoretic study of the blood serum from lymphogranulomatous patients 624
- ROWE, PETER AND SHELTON JOHN M Synthetic diets, 1059
- RUBIN SAUL H (See PARSONNET BERNSTEIN KLOSK HIRSCHBERG RUBIN AND PIRK), 602
- RUBINSTEIN MICHAEL A Transient positive Wassermann test for syphilis in acute hemolytic anemia 753
- RUBY BARBARA (See LANDOWNE, THOMPSON AND RUBY), 1482
- RICHMAN ISAAC Occurrence of toxoplasma neutralizing antibodies in various disease conditions 87
- RITLEDGE ROBERT C, JR. The spectrophotometric determination of blood pH, 881
- RYAN EDWARD J AND MORGAN, PHILIP W Psychosomatic problems in a private practice 1621
- RYAN JEAN TURNBULL (See MOUS FREDERIC E), 392
- S
- SALASCA ROBERT M, BOLLMAN JESSE L AND DRY THOMAS J The effect of para-aminobenzoic acid on the metabolism and excretion of salicylate 1393
- SAMPATH ANUS (See LITTLE SAMPATH, AND SUBBAROW), 1144
- SASLAW SAMUEL (See CAMPBELL AND SASLAW), 1207
- AND CAMPBELL CHAILOTTE C The use of yeast phase antigens in a complement fixation test for histoplasmosis I Preliminary results with rabbit sera 811
- SANDERS GEORGE M TALMAGE DAVID W AND SCOTT VIRGIL The use of Plasmodium vivax preserved by freezing in inducing malaria 1579
- SAWITSKY ARTHUR (See MEYER SAWITSKY RITZ AND FITCH), 189
- FITCH HOWARD M AND MEYER LEO M A study of cholinesterase activity in the blood of normal subjects 203
- SCANLON MARGARET H (See METCOFF DARLING SCANLON AND STARE), 47
- SCHEM, F R AND LAYNE, J A The effect of water by vein on the more severe complications of cardiovascular renal disease, 1467
- SCHICK ARMIN AND HISS, GEORGE M The preparation and properties of mammalian striated myofibrils 1612
- SCHIFF L (See COGSWELL CLEVELAND GALL WEISBROD AND SCHIFF), 1627
- SCHLESINGER ROBERT B HARDT, LEO L AND STEIGMANN FRIEDRICH Diagnostic and therapeutic implications of acidity variations in the stimulated and nonstimulated stomach 1626
- SCHLICHTER J G Etiology of auricular fibrillation and the mechanism of its perpetuation 1492
- SCHNEIDERSON S STANLEY (See JANOWITZ SCHNEIDERSON, SUSSMAN AND KING), 933
- SCHOENBERGER JAMES (See LOOSLI SCHOENBERGER AND BARNETT), 789
- SCHOOMER MARY LYNN (See MAY BENNETT LANE FUTCH SCHOOMER AND GREGORY), 1494
- SCHRAM, WARREN R (See RHOADS AND SCHRAM), 1461

- SCHROEDER, HENRI A, AND GOLDMAN, MELVIN L A test for the presence of the hypertensive diencephalic syndrome using histamine, 1470
- SCHPOEDER, MADELINE S (See GUTERMAN AND SCHPOEDER), 356
- SCHUELER, F W The effect of succinate in mescaline hallucinations, 1297
- SCHWARTZ, B S, AND LEWIS, M N A practical method for the preparation of organs for the determination of antibiotic content, 904
- SCHWEIGERT, B S (With the technical assistance of PANZER, FRANCIS, AND SPARKS, PATRICIA) Folic acid metabolism studies III Intravenous administration of pteroylglutamic acid and pteroyltriglutamic acid, 1271
- SCOTT, ROY W, AND ZIMMERMAN, HENRY A A direct writing ossilograph for recording data obtained by the method of cardiac catheterization, 1499
- SCOTT, VIRGIL (See SAUNDERS, TALMAGE, AND SCOTT), 1579
- SCUDAMORE, H H (See VORHAUS, SCUDAMORE GABUZZI, MOREY, MALONEY, AND KARK), 1475
- SCUDI, JOHN V, AND REINHARD, JOHN F Absorption, distribution, and renal excretion of mandelamine (methanamine mandelate), 1304
- SEELEY, SAM F (See PULASKI AND SEELEY), 1
- SEGALOFF, ALBERT (See DAVIS, SEGALOFF, AND JACOBS), 1483
- SHANBERGE, JACOB N (See QUICK, SHANBERGE, AND STEFANINI), 1424, 1479
- SHANER, GRACE A (See BOGER, MILLER, TILLSON, AND SHANER), 297
- SHARP, ELWOOD A (See BERNAN, AXELROD, JACOBSON, HORAN, VONDERHEIDE, AND SHARP), 1628
- (See BERNAN, AXELROD, VONDERHEIDE, AND SHARP), 1643
- SHATZ, BURTON A, BERGMANN, MARTIN, AND GRAY, S H Sputum cell study for pulmonary carcinoma as a routine laboratory test, 1588
- SHAW, PHYLLIS (See BOHLS AND SHAW), 239
- SHEETS, RAYMOND F (See HAMILTON, SHEETS, AND DEGOWIN), 1650
- SHELBURNE, SAMUEL A Retinal arteriovenous nicking II A long term study of the development of arteriovenous nicking in patients with hypertension, 1486
- SHELDON, JOHN M (See ROWE AND SHELDON), 1059
- SHEN, SHU CHU (See WAGLEY, SHEN, GARDNER, AND CASTLE), 1197
- SHIPLEY, R E (See HELMER, SHIPLEY, PEIRCE, AND KOHLSTAEDT), 1484
- SHULMAN, BERNARD H (See STEIGMANN, POPPER, AND SHULMAN), 1630
- SIEGEL, LEO H, AND KRAEMER, MANFRED Further studies on the Weltmann serum coagulation reaction, 618
- SILBERSTEIN, JOSEPH K, BERNSTEIN, THEODORE C, AND STERN, THELMA Demonstration of heterophile antibodies in the cerebrospinal fluid from patients with infectious mononucleosis, 1204
- SIMS, J L Antidiuretic activity of urine in acute hepatitis, 1476
- SINGAR, KARL, KING, JOSEPH C, AND ROBIN, SIDNEY The life span of the megakaryocyte and the hemolytic syndrome of pernicious anemia, 1068
- , ROBIN, SIDNEY, KING, JOSEPH C, AND JEFFERSON, RONALD N The life span of the sickle cell and the pathogenesis of sickle cell anemia, 975
- SINCER, RICHARD B (See ORDWAY AND SINCER), 511
- SLAUGHTER, DANLEY (See TAYLOR, SLAUGHTER, PRESTON, CRUMBINE, AND HASS), 1645
- SLAUGHTER, OWEN L, BROWN, HUGH S, AND WAHIM, KHALIL G Effects of tetraethylammonium chloride on blood flow in the extremities of man, 743
- SMITH, MARTIN H, AND HALL, JAMES W Infectious hepatitis inadvertently transmitted with therapeutic malaria, 998
- SMITH, RALPH O, AND WOOD, W BARRY, JR The pathogenesis of acute bacterial lymphadenitis, 1458
- SMITH, ROBERT E, STEELE, J MURRAY, EAKIN, ROBERT E, AND COWIE, DEAN B The tissue distribution of radioantimony inhaled as stibine, 635
- SMITH, TAYLOR R (See BLOCK, JACOBSON, NEAL, AND SMITH), 1649
- SMULL, KATHARINE, WISSLER, ROBERT W, AND WATSON, JAMES M The effect of sodium salicylate upon serum disease in rabbits, 936
- SMYTHE, RICHARD L (See MANN, GEYER, WATKIN, SMYTHE, ZANCHECK, AND STARE), 1503
- SNARELY, JOHN G (See GOLDEN AND SNARELY), 890
- SODEMAN, W A Emetine therapy in the presence of heart disease, 1457
- SOLOMON, WALTER M, AND STECHER, ROBERT M Antireticular cytotoxic serum in the treatment of arthritis Observations on forty four cases, 768
- SPARKS, PATRICIA (See SCHWIGERT, B S), 1271
- SPFAR, FRANCES Determination of antibody content of lymphoid cells extracts, 1563
- SPICEF, SOPHIE, AND BLITZ, DIANA A study of the response of bacterial populations to the action of penicillin, a quantitative determination of its effect on the organisms, 417
- SPIES, TOM D (See STONE AND SPIES), 1019
- SPRAGUE, JAMES M (See ZIFGLEF AND SPRAGUE), 96
- SPRAGUE, R G, HAYLES, ALVIN B, MASON, H L, POWER, M H, AND BENNETT,

- W 1 "Steroid diabetes associated with Cushing's syndrome and excretion of 17 hydroxycorticosterone (compound F) in urine: metabolic studies 1472
- STARE, FREDRICK J (See COLLINS KRAFT KINNEY, DAVIDSON YOUNG AND STARE) 143
- (See GEYER MARY AND STARE) 137 175
- (See GEYER MARY YOUNG KINNEY AND STARE) 163
- (See MANN AND STARE) 1161
- (See MANN GEYER WATKIN SMITH ZAMCHECK, AND STARE) 1003
- (See METCOFF, DARLING SCANLON AND STARE, 47
- STECHEE, ROBERT M (See SOLOMON AND STECHER) 768
- STEELE J MURRAY (See SMITH STEELE EAKIN AND COWIE) 635
- STEFANINI MARIO (See QUICK AND STEFANINI) 819
- (See QUICK SILANBERGE AND STEFANINI) 1424 1479
- STEFFEE, C HAROLD (See BENDITT HUN PHREYS WISSLER STEFFEE FRAZIER AND CANNON) 257
- STEIGMANN FREDERICK (See FRANKLIN POPPER STEIGMANN AND KOZOLL) 435
- (See POPPER STEIGMANN DANIELS AND DUBIN) 1630 1631
- (See SCHLESINGER, HARPT AND STEIGMANN) 1626
- , POPPER HANS AND SHULMAN BERNARD H Combination of flocculation tests in the differential diagnosis of jaundice 1630
- ZWIG MITCHELL AND MEYER KARL A Gastric acidity response during the intravenous administration of protein hydrolysates 1627
- STERTO ROBERT (See BENDITT WOODRIDGE AND STERTO) 269
- STEPAN KURT G (See ROTTINO SUCHOFF AND STERN) 624
- STERN, THELMA (See SILBERSTEIN BERNSTEIN AND STERN) 1204
- STICKNEY J M HAGEDORN A B MILLS S D AND COOPER TALBERT Changes in blood and bone marrow in acute leucemia induced by aminopterin 1481
- STILLERMAN, HYMAN S The thymol turbidity test in various diseases 665
- STOKSTAD E L R PAGE, A JR ILICF J FRANKLIN A L JUKES T H HEINLE R W ELSTEIN M AND WELCH A D Activity of microbial animal protein factor concentrates in pernicious anemia 860
- STONE ROBERT E AND SPIES TOM D The effect of liver extract and vitamin B<sub>12</sub> on the mucous membrane lesions of macrocytic anemia 1019
- STRAUMFJORD JOV V (See VAN BRUGGEN AND STRAUMFJORD) 67
- STRAUSS J JR AND NACHELES H Variations in dermal absorption with age, 612
- STEICHER MICHAEL H The chemical evaluation of duodenal substance in chronic ulcerative colitis 1633
- STIMMA MAX M AND HERSHEY J IVAN Imbedding of pathologic specimens in transparent plastic, 1311
- SUBAROW Y (See HARVED CUNNINGHAM HALLIDAY VESSEY YUDA CLARK HINE COSGROVE AND SUBAROW) 216
- (See LITTLE OLESON AND SUBAROW) 119
- (See LITTLE SAMPATH AND SUBAROW) 1144
- (See MCKENZIE MALONE KUSHNER OLFSON AND SUBAROW) 1249
- SUCHOFF DIANA (See ROTTINO SUCHOFF AND STERN) 624
- SUSSMAN MARCY L (See JANOWITZ SCHNEIDERSON SUSSMAN AND KING) 933
- SUTTON GEORGE C WENDEL GEORGE E AND GRANT HARRY E A comparative study of peripheral and direct intracardiac angiocardiology 1502
- — — AND WARNICK EDWARD G Circulation times for angiocardiology, 1501
- SWAITS JEROME M BERNSTEIN ARTHUR RAGINS ALEX B AND MEYER JACOB The cytologic diagnosis of cancer of the stomach preliminary report 1033

## T

- TAYMAGE DAVID W (See SAUNDERS TAYMAGE AND SCOTT) 1579
- TALSO PETER J CROSBY ARCHER P JR AND CLARKE ROBERT W Effects of the cold pressor test on glomerular filtration and effective renal plasma flow 420
- TATTING BETTY (See CARTWRIGHT FAY TATTING AND WINTROBE) 397
- TAYLOR C BRUCE HASS GEORGE M AND MALONEY JOHN E Relations between volumes of closed hypothermal cerebral lesions and symptoms in rabbits 1462
- TAYLOR ROBERT D CORCORAN A C FERTIG H H AND PAGE IRVINE H The effects of bacterial pyrogens on malignant hypertensive persons 1469
- TAYLOR SAMUEL G III SLAUGHTER DAVID PRESTON FREDERICK W CRUM RINE JOSEPH AND HASS GEORGE Effect of antifolate acid derivatives on patients with far advanced carcinomas 1645
- THAYER JAMES D (See MANDL AND THAYER) 135

- THOMAS, GEORGE E, AND KITZBERGER, DORIS M A spectrophotometric method for determination of urinary bilirubin, 1189
- THOMAS, E M (See WOLFSON, COHN, CALVARY, AND THOMAS), 1276
- THOMAS, JAMES D (See WRIGHT, DOAN, DODD, AND THOMAS), 708
- THOMAS, THEODOPE B (See MOSES, CRITCHFIELD, AND THOMAS), 448
- THOMPSON, FRANCES L (See COHN AND THOMPSON), 75
- THOMPSON, ROBERT T Antihyaluronidase antagonistic to pneumococcus hyaluronidase in the serum of normal human beings and patients with pneumococcal pneumonia rise of titer in bacteremic pneumococcal pneumonia, 919
- THOMPSON, WALTER S (See LANDOWNE AND THOMPSON), 1609
- (See LANDOWNE, THOMPSON, AND RUBY), 1482
- TILLSON, ELIZABETH (See BOGER, MILLER, TILLSON, AND SHANER), 297
- TOBIAS, C A (See BERTRAND, WAINE, AND TOBIAS), 1133
- TOBIAS, CHARLES W (See ROSENTHAL AND TOBIAS), 1110
- TOMPKINS, EDNA H Factors in the use of mercuric bichloride for biologic studies, with especial reference to blood count, 1094
- Methods to increase accuracy in the use of Hayem's solution for red blood counts, 1180
- TREPTOW BETTY (See DIECKMANN, PRIDDLE, TURNER, AND TREPTOW), 1641
- TRIMBLE, GEORGE X Ventricular irregularities induced by symptho-adrenal discharge and chloroform, 1438
- TRUSSELL, RAY E (See KORN AND TRUSSELL), 1150
- TURNBULL, GEORGE C (See NARUT AND TURNBULL), 1468
- AND BRADEN, EDWIN S, JR Bleeding peptic ulcer, a report of one hundred and sixty cases, 1624
- TURNER, ROSE (See DIECKMANN, PRIDDLE, TURNER, AND TREPTOW), 1641
- V
- VALENTINE, WILLIAM N (See LAWRENCE, VALENTINE, AND ADAMS), 1077
- VAN BRUGGEN, JOHN T A comparison of methods used for the hydrolysis of conjugated urinary estrogens, 207
- , AND STRAUMFJORD, JON V High vitamin A intake and blood levels of cholesterol, phospholipids, carotene, and vitamins C, A, and E, 67
- VAN HOOK, EMEY (See BOLLMAN, JESSE L), 1348
- (See BOLLMAN, CAIN, AND GRINDLAY), 1349
- VESSEY, R E (See HARNED, CUNNINGHAM, HALLIDAY, VESSEY, YUDA, CLAPH, HINE, COSGROVE, AND SUBBAROW), 216
- VILTER, CARL F (See MITHOEFFER AND VILTER), 1471
- VONDERHEIDE, ELMORE C (See BERMAN, AVELROD, JACOBSON, HORAN, VONDERHEIDE, AND SHARP), 1628
- (See BERMAN, AVELROD, VONDERHEIDE, AND SHARP), 1643
- VORHAUS, L J, SCUDAMORE, H H, GABUZDA, G J, MOREY, G R, MALONEY, M A, AND KARK, R M Serum cholinesterase determinations in liver disease, 1475
- W
- WADE, LEO, NEUDOFF, LOUIS, FRITZ, HERBERT, AND KARL, MICHAEL The effect of choline, methionine, and low fat diet on the life expectancy of patients with cirrhosis of the liver, 1123
- WAGENER, HENRY P (See HORTON AND WAGENER), 1611
- WAGLEY, PHILIP F, SHEN, SHU CHU, GUDNER, FRANK H, AND CASTLE, WILLIAM B Studies on the destruction of red blood cells VI The spleen as a source of a substance causing agglutination of the red blood cells of certain patients with acquired hemolytic jaundice by an antihuman serum rabbit serum (Coombs' serum), 1197
- WAINE, H (See BERTRAND, WAINE, AND TOBIAS), 1133
- WAKIM, KHALIL G (See BEFREY, BALDES, ESSEX, AND WAKIM), 101
- (See HAYES, WAKIM, HOFTON, AND PETERS), 1497
- (See SLAUGHTER, BROWN, AND WAKIM), 743
- WALLACE, STANLEY L, LITTLE, J MAXWELL, AND BOBB, J R R The relation of phenol retention to uremia and the effect of phthalylsulfathiazole and streptomycin on phenol production, 845
- WALLER, WILLIAM S (See PECK AND WALLER), 784
- WANG, C F, LAPE, A, AND HEGSTED, D M Studies on the minimum protein requirements of adult dogs, 462
- WARNER, E D (See BOYD AND WARNER), 1431, 1651
- WAPNICK, EDWARD G (See SUTTON, WEADEL, GRANT, AND WAPNICK), 1501
- WATKIN, DONALD M (See MINN, GEYER, WATKIN, SMYTHE, ZAMCHECK, AND STARE), 1503
- WATKINS, CHARLES H (See DILL AND WATKINS), 480, 487
- WATSON, CECIL JAMES (See STICKNEY AND WATSON), 542

- WATSON JAMES M (See SMULL WISSLER, AND WATSON), 936
- WEAVER, R H (See ARNOLD AND WEAVER), 1354
- (See HANNAH AND WEAVER) 1378
- WEBB, CELLA R (See BRIAN WILLIAMS AND WEBB) 1678
- WEISBERG F (See COGSWELL CLEVELAND GALL WEISBERG AND SCHIFF) 1621
- WEISMAN, S A (See LEIGIN AND WEISMAN) 1492
- Experimental studies on the combined action of digitals (cedilana) and quinidine (in cats) 1493
- WEISBERG, JOHNS (See MCGIVACI, WEISBERG AND BOYD), 595
- WEICH, ARNOLD D (See HEINLE WEICH AND PRITCHARD) 1647
- (See STOKSTAD PACE HERCE FRANKLIN JUKES HEINLE EPSTEIN AND WELCH), 800
- WELCH, HENRY (See HIESH, WELCH MILL OFF AND KATZ) 503
- WELCHER MERRILL L (See HAMMOND AND WELCKER), 1354
- WENDEL, GEORGE E (See SUTTON WENDEL AND GRANT), 1502
- (See SUTTON WENDEL, GRANT AND WAINICK), 1501
- WERNER HAROLD W (See BROWN AND WERNER) 325
- WESSELS K E (See BOYD, CHEYNE AND WESSELS), 1614
- WHEELER CLAYTON E (See CONY LOLIS AND WHEELER) 651
- WIEGAND HERBERT C (See MASSIE AND WIEGAND), 1487
- WIENER, ALEXANDER S Intragroup incompatibility with respect to the H<sub>r</sub> blood factors as a cause of minor hemolytic transfusion reactions, 985
- AND GORDON EVE B Studies on the coagulation test in erythroblastosis fetalis, 181
- WILKERSON, HUGH L C AND HEFTMANN LEIGH Screening method for blood glucose 236
- WILLIAMS M M D (See CHILDS, KEATING WILLIAMS AND POWER) 1615
- WILLIAMS T FRANKLIN (See BRIAN WILLIAMS AND WEBB), 1638
- WILSON, KEITH S (See BURANTZ, DAMIAN WILSON, AND JOHNSON) 1463
- WILSON, RUSSELL H (See BORDEN, WILSON AND EBERT) 1453
- WINTROBE MAXWELL M (See CARTWRIGHT FAY, TATTING AND WINTROBE) 397
- (See MUNDER AND WINTROBE) 332
- WISEMAN BRUCE K (See BROWN READ WISEMAN AND FRANCE) 1623
- WISSLER, ROBERT W (See BENDITT HILM PHREYS, WISSLER STEFFEE FRAZIER AND CANNON) 257
- (See SMULL WISSLER AND WATSON) 936
- WITEBSKY ERNEST (See MOHN AND WITTEBSKY), 1361 1369
- AND MOHN, JAMES F Studies on Rh antibodies I Analysis of a zone phenomenon in an Rh antiserum by splitting the serum into two fractions by means of dialysis 1553
- WOLFE HAROLD G (See BLANKENHORN AND WOLFE) 1165
- WOLFSON WILLIAM QUITMAN (See CONY AND WOLFSON) 367
- CONY C CALHUN E AND THOMAS E M Studies in serum proteins IV Chemical studies employing rapid chemical fractionation procedures, with particular reference to the frequency and significance of hypoalbuminemia 1276
- WOLNIK B (See DOBART HALPERN KUTZ, LESIE WOLNIK AND DEY), 1608
- WOOD EARL H (See CROOK, WOOD AND ODGL) 167
- WOOL W BURR JR (See SMITH AND WOOD) 1459
- WOOLFORD, NORMA (See LEECH AND WOODFORD) 644
- WOOLFIDE ROBERT L (See BENDITT WOOLFIDE AND STEPTO) 269
- WRIGHT CLAUDE STARR, DOAN, CHARLES A, DODD VERNE A, AND THOMAS JAMES D Hemophilia current theories and successful medical management in traumatic and surgical crises 708
- Y
- YOUNG JOHN (See COLLINS KRAFT HENRY DAVIDSON YOUNG, AND STARE) 14
- (See GEYER MARY YOUNG KINNEY AND STARE) 163
- YUDA, N N (See HAF ED CUNNINGHAM, HALLIDAY VESSEY YUDA, CLARK HIVE COSGROVE AND SUBBAROW) 216
- Z
- ZAMCHECK NORMAN (See MARY GYER, WATKIN SMYTHIE ZAMCHECK AND STAFF) 1603
- ZAVES ROBERT P JR DOAN CHARLES A, AND HOSTER HERMAN A Studies in Hodgkin's syndrome VII Nitrogen mustard therapy 1002
- ZIRAFOVETIS CHRIS J D Paraaminobenzoic acid therapy in scleroderma and lymphoblastoma cutis 1462
- ZEIT WALTER (See EVANS AND ZEIT), 1454
- ZHEUTLIN HAROLD E C (See RAYMOND AND ZHEUTLIN) 1193
- ZIEGLER, CARL AND SPRAGUE JAMES M A colorimetric determination of caronamide 96
- ZIMMERMAN HENRY A (See SCOTT AND ZIMMERMAN) 1499
- ZUCKERMAN JOSEPH L AND NITELSON SAMUEL A convenient and rapid procedure for total cholesterol estimation using an acid chloroform extraction 1322
- ZWEIG MITCHELL (See STEIGMAN; ZWEIG AND MEYER) 1627

## SUBJECT INDEX

### A

- Acetylcholine, sensitivity of heart to, effects of acute hypoxia on, 1495
- Acidity, gastric, response during administration of protein hydrolysates, 1627
- variations in stimulated and nonstimulated stomach, diagnostic and therapeutic implications of, 1626
- Actinomycosis, penicillin in treatment of, 521
- Addisonian pernicious anemia, effect of vitamin B<sub>12</sub> on hematopoietic and nervous systems in, 1646
- Adrenal cortex, role of, in urate metabolism and in gout, 1473
- Adrenalectomy and adrenal cortical hormone, influence of, on hypoferrinemia and other blood changes associated with injection of turpentine, 532
- Adrenalin in normal and hypertensive patients, effects of, in relation to mechanism of sustained pressure elevations, 332
- Albumin and globulin in serum or plasma, determination of, a rapid clinical method for the accurate, 367
- egg, increase of complement units by, 239
- globulin, and hemoglobin contents in human, micromethod for determination of, 1440
- human, clinical method for determination of, by means of a precipitin reaction, 1052
- Alcoholic fatty liver, observations on, with use of interval needle biopsy and liver function tests, 555
- Allergy, food, acute torticollis due to, 1614
- Alloxan, diabetic response of weanling rats to intravenous doses of, 1161
- Amino acid preparation, a new lyophilized, determination of nitrogen balance index of, in protein deficient patients, 280
- Aminopterin, acute leucemia induced by, changes in blood and bone marrow in, 1481
- treatment of acute leucemia with, 1642
- Anemia, acute hemolytic, transient positive Wassermann test for syphilis in, 753
- Addisonian pernicious, effect of vitamin B<sub>12</sub> on hematopoietic and nervous systems in, 1646
- aplastic, role of blood and bone marrow concentrations of itabrine in, 827
- macrocytic, mucous membrane lesions of, effect of liver extract and vitamin B<sub>12</sub> on, 1019
- of infection, 532
- of pregnancy treated with molybdenum iron complex, 1641
- pernicious, activity of microbial animal protein factor concentrates in, 860
- cholinesterase activity of blood in, 189
- Anemia, pernicious—Cont'd
- life span of the megalocyte and the hemolytic syndrome of, 1068
- vitamin B<sub>12</sub> in, 1477
- puerperal macrocytic, vitamin B<sub>12</sub> in, 1477
- sickle cell, pathogenesis of, and the life span of the sickle cell, 975
- Anesthetized dogs, tidal volume recorder for, 784
- Angina pectoris, electrocardiographic changes following meals in patients with, 1501
- Angiocardiography, circulation times for, 1501
- peripheral and direct intracardiac, comparative study, 1502
- Animal protein factor concentrates, activity of microbial, in pernicious anemia, 860
- Anoxemia test in hyperthyroidism, electrocardiographic response to, 1500
- Anterior pituitary extract in treatment of pituitary dwarfism, evaluation of, 1615
- Anti A and anti B isoagglutinin titers in Rh immunized pregnant women, 1089
- Antibiotic content, determination of, practical method for preparation of organs for, 904
- Antibodies against renin and "sustained pressor principle" produced by kidney extracts, 1484
- toxoplasma, neutralizing, occurrence of, in various disease conditions, 87
- Antibody content of lymphoid cell extracts, determination of, 1563
- formation following ingestion of heat killed *Brucella abortus*, 1459
- Anticoagulant drugs, effect of, upon coronary flow, 1455
- therapy, use of intramuscular heparin for, 1652
- Antifolic acid derivatives, effect of, on patients with far advanced carcinoma atosis, 1645
- Antigen, cardiolipin, studies on, 1220, 1224
- for Kahn test, standardization of, 1224
- Antigenic differences among influenza A viruses, 1212
- Antigens, yeast phase, in complement fixation test for histoplasmosis, 811, 1207
- Antihistaminic agent, neohetramine a new, experimental and clinical studies of, 865
- drug, decapryn succinate, experimental and clinical results, 319
- 2 [ $\alpha$  (2 dimethylaminoethoxy)  $\alpha$  methyl benzyl] pyridine succinate (decapryn succinate), experimental and clinical results, 319
- drugs, anesthetic and antihistaminic action of a series of, in human skin, 1609



Antihistaminic drugs—Cont d  
 failure of to reduce reactive hyperemia in man 1609

Antihyaluronidase antagonistic to pneumococcus hyaluronidase in serum of normal human beings and patients with pneumococcal pneumonia 919

Antireticular cytotoxic serum in treatment of arthritis 768  
 failure of 1.89

Antitoxin diphtheria and tetanus purification of, by use of pepsin 757

Anuria experimental, dietary and hormonal influences in 1489

Apparatus for producing quantitative ischemia in limbs of animals 1450

Arterioles of skin in essential hypertension 1486

Arthritis antireticular cytotoxic serum in treatment of, 768  
 distribution of gold in animal body in relation to, 1133  
 failure of antireticular cytotoxic serum in 1289  
 gold compounds in treatment of metubolism, toxicity and manner of action of 1381

Ascorbic acid glycosuria simulated by the administration of occurrence and differentiation 1471

Asthma, action of pilocarpine on lungs in 385

Atabrine, blood and bone marrow concentration of and its role in aplastic anemia 827

Atherosclerosis development of in chickens on normal and low fat diet effect of diethylstilbestrol on blood lipids in 733

Auricular fibrillation dicumarol and quinidine in ambulatory treatment of chronic 1492  
 etiology of and the mechanism of its perpetuation 1492

B

Bacteremia following tooth extraction prevention with penicillin and gentrocan 1461

Bacteremic pneumococcal pneumonia rise of titer in 919

Bacteria streptomycin resistant development of in stools of patients treated for tuberculosis 1460

Bacteria induced shock resulting from crushing of muscle of dogs mechanism of 502

Bacterial lymphadenitis acute pathogenesis of 1458  
 populations, response of to action of penicillin 417  
 pyrogens effects of on malignant hypertensive persons 1469

BIL and other thiol compounds effect of in preventing the inhibition of oxygen consumption of rat tissues produced by gold salts 1381  
 effect of on gold toxicity in rats 1024

Barium diphenylamine sulfonate used as indicator for chromic acid titration in determination of volatile reducing substances in blood and gases 126

Bedrin deaths 1457

Bedside method for estimation of blood sugar, a simple 644

Benadryl and pyribenzamine comparison of toxic manifestations produced by 595  
 rutin and protamine influence of on experimental purpura in the guinea pig 1651

Berylliosis clinical study 1613

Beta glucuronidase activity of human endometrium 1619

Beta dimethylaminoethyl benzhydryl ether hydrochloride and tripeptamine comparison of toxic manifestations produced by 595

Bilirubin in urine quantitative determination of method for 890

Biologic fluids ethanol in colorimetric method for determination of microquantities of, 241  
 sodium in human, microcolorimetric determination of 246  
 materials for common steam volatile or gaseous poisons systematic qualitative analysis of 371  
 tissue radioactive isotopes from recovery and estimation of 1169

Biopsy needle of liver in hepatic cirrhosis experiences with 1627  
 use of interval in observations on alcoholic fatty liver 535

Blood and bone marrow changes in in acute leucemia induced by aminopterin, 1481  
 concentration of atabrine and its role in aplastic anemia 827  
 in cirrhosis of liver 1628  
 and urine lactate influence of physical fitness and convalescence from major surgical procedures on the magnitude of the responses to exercise of 1474

cells red destruction of studies on 1197

cholesterol and arterial intima in rat, effect of feeding propylthiouracil and cholesterol on 1029

cholinesterase activity in normal 203  
 in pernicious anemia 189

clot retraction effect of operation and illness on description of new method 347

coagulability effect of digitals on 1653

coagulation effect of nitrogen mustard and X irradiation on 1566

concentration of labile factor of prothrombin complex in human dog and rabbit significance in determination of prothrombin activity 819

dyscrasias serum proteins of electrophoretic analysis of 1523

ethanol in colorimetric method for determination of microquantities of 241

fat in after intravenous administration of fat emulsions turbidimetric determination of 175

## Blood—Cont d

- flow, effects of tetraethylammonium chloride on, in extremities of man, 743  
in human extremities, compensating plethysmograph for measuring, 101  
of dog, coronary, effect of certain sympatholytic agents on, 1496  
glucose, screening method for, 236  
heparinized in vitro, coagulation time of, test of, studies of normal subjects and those with intravascular thrombosis, 1342  
human, measurement of electric resistance of, use in coagulation studies and cell volume determinations, 1110  
iodine levels of man, influence of oxyquinoline drugs on growth of *Endamoeba histolytica* as measured in, 1632  
levels, high vitamin A intake and, of cholesterol, phospholipids, eucotene, and vitamins C, A, and E, 67  
lipids, effect of diethylstilbestrol on, and on the development of atherosclerosis in chickens on a normal and low fat diet, 733  
penicillin levels achieved with caronamide, role of high, in penetrating the blood brain barrier, 933  
peripheral, appearance of normoblasts in patients with pulmonary embolism, 1502  
pH, spectrophotometric determination of, 881  
pressure curves, intra-uterine, during tilting, 1483  
effects of splanchicectomy on, and on cardiac and renal function, 1485  
maintenance in normal and toxemic pregnancy using tetraethylammonium chloride, evaluation of neurogenic and humoral factors in, 1466  
radial or brachial, intra-arterial, alterations of, and of electrocardiogram induced by tilting, 951  
relation of fluid intake to, 853  
red counts, Hygem's solution in, methods to increase accuracy in use of, 1180  
serum, electrophoretic study of, from lymphogranulomatous patients, 624  
sugar, estimation of, simple bedside method for, 644  
transfusion, direct, failure of, to raise the platelet level, 1077  
volatile reducing substances in, determination of, using barium diphenylamine sulfonate as an indicator for chromic acid titration, 126
- Blood brain barrier, role of high blood penicillin levels achieved with caronamide in penetrating, 933
- Body fluids, examination of, a modified technique for, 1195  
surface temperature of, thermocouples for measurement of, improved mounting for, 1044
- Bone marrow, cellular composition of, in normal infants and children, 1639
- Bromsulfalein test of liver function, importance of rate of dye removal in, 448
- Brucella abortus, antibody formation following ingestion of heat killed, 1459
- C
- Cage limiting activity of rats, 1348
- Cages containing cotton rats, device to facilitate the cleaning of, 519
- Calcification, diffuse pancreatic, with diabetes mellitus, 1619  
of costal cartilages, premature, association of, with psychoneuroses and endocrine imbalance, 1631
- Cancer, microscopically controlled excision of, preparation of frozen sections for use in chemosurgical technique for, 392  
of stomach, cytologic diagnosis of, 1633
- Capillaries, thermal injury on, effect of various lethal procedures and, 497
- Carcinoma, pulmonary, sputum cell study for, as routine laboratory test, 1588
- Carcinomatosis, far advanced, effect of antifolic acid derivatives on patients with, 1645
- Cardiac catheterization, direct writing oscillograph for recording data obtained by method of, 1499  
decompensation, qualitative and quantitative definition of cations and anions in, 1534  
glycosides, action of several, on excitability and conduction velocity in dog heart, 1491  
output, effect of apprehension caused by technical procedures on, 1494  
sympathectomy, paroxysmal tachycardia benefited by, 1495
- Cardiolipin antigen, studies on, 1220, 1224
- Cardiorespiratory function, studies on, with the oxymograph, 1454
- Cardiovascular renal disease, complications of, effect of water by vein on the more severe, 1467
- Caronamide, colorimetric determination of, plasma concentration, urinary recoveries, and dosage, 297
- Carotene, high vitamin A intake and blood levels of, 67
- Casein hydrolysate, oral administration of, effect of, on total circulating plasma proteins of man, 453
- Castor bean sensitivity, 1613
- Catheterization, venous, with polyethylene tubing, 1597
- Central Society for Clinical Research, president's address, October 29, 1948, 1458  
twenty first annual meeting, Chicago, Ill., October 29 and 30 1948, transactions of, 1451, 1606
- Cephalin cholesterol flocculation and thymol turbidity tests in serum, a dilution turbidity test in comparison with, 672  
test, serum albumin globulin ratio, and thymol turbidity test for liver function, comparative study, 662

- Cerebral lesions, volumes of closed hypothermal, relations between, and symptoms in rabbits 1402
- Cerebrospinal fluid heterophile antibodies in infectious mononucleosis 1204
- Chemosurgical technique for microscopically controlled excision of cancer preparation of frozen sections for use in 392
- Chemotherapy of filariasis 216
- Chlorine effect of on complement fixation 204
- Cholesterol estimation total convenient and rapid procedure for using in acid chloroform extraction 1322
- high vitamin A intake and blood level of 67
- tolerance tests in normal diabetic and hypertensive patients 1619
- Choline methionine and low fat diet effect of on life expectancy of patients with cirrhosis of liver 1123
- Cholinesterase activity of blood 203
- in pernicious anemia 189
- determinations in liver disease serial serum 1475
- Circulation time accurate indicator for objective measurement 910
- for angiocardiology 1501
- Cirrhosis of liver blood and bone marrow in 1628
- life expectancy of patients with effect of choline methionine and low fat diet on 1123
- Cleaning of cages containing cotton rat device to facilitate the 519
- Clinical erythroblastosis fetalis in newborn maternal isoimmunization without evidence of 526
- Clot retraction, effect of operation and illness on, description of new method 347
- Congulability of blood effect of digitalis on 1653
- Congulation defect in thrombocytopenic purpura 1479
- time of blood heparinized in vitro test of study of normal patients and those with intravascular thrombosis 1342
- Cold hemagglutinin test by slide method 144
- pressor test effects of on glomerular filtration and effective renal plasma flow 430
- Colitis ulcerative duodenal substance in chronic clinical evaluation of 1633
- Colorimetric determination of caronamide 96
- estimation of pregnandiol in urine simplified technique for the quantitative 356
- method for determination of microquantities of ethanol in blood and other biologic fluids 241
- of pregnandiol a new 251
- Complement fixation, effect of chlorine on 254
- test for histoplasmosis use of yeast phase antigens in 811 1207
- Complement fixation test—Cont d
- for syphilis quantitative method for determination of titers between 10 and 100 in 383
- units increase of egg albumin 239
- Concentration of fibrin blood and bone marrow and its role in aplastic anemia 827
- of fibrile factor of prothrombin complex in human dog and rabbit blood, significance in the determination of prothrombin activity 919
- Conglutination test in erythroblastosis fetalis, 141
- Coombs serum acquired hemolytic jaundice by spleen as source of substance causing agglutination of red blood cells in 1197
- Coronary blood flow of dog effect of certain sympatholytic agents on 1496
- flow effect of anticoagulant drugs upon 1450
- Corticosteroids chemical determination of in urine and plasma methods for, 1321
- Cotatal antigens premature calcification of its association with psychoneuroses and endocrine imbalance 1621
- Crytalline hemin solutions as permanent standards for hemoglobin estimation 83
- Crytillization of serotonin 1606
- Cultures identification of quick microtechniques for 1334 1338
- Cushing's syndrome steroid diabetes associated with and excretion of 17 hydroxycorticosterone (compound F) in urine, metabolic studies 1472
- Cytochrome C intravenous administration in man 294

## D

- Decaprin succinate new antihistaminic drug experimental and clinical results of 319
- Delayed death mechanism of following thermal trauma 506
- Dental caries in an institutional population correlation of lactobacillus counts with extent of, 1614
- Dermal absorption variations in with age, 612
- Desoxycorticosterone acetate and propylene glycol effect of in experimental renal hypertension 1493
- action mechanisms of 855
- Diabetes cholesterol tolerance tests in 1618
- experimental nutrition and 1161
- mellitus diffuse pancreatic calcification with 1619
- production of temporary with pituitary adrenocorticotrophic hormone, relation to uric acid metabolism 651
- tubular reabsorption of glucose in effect of insulin on 1616
- Dibenamine 1496
- Dicummarol and heparin, comparative action on, the in vivo clot experimental study of 731

- Dicumarol—Cont'd  
 and quinidine in ambulatory treatment of chronic auricular fibrillation, 1492  
 effects of, on electrocardiogram, 972  
 in experimental myocardial infarction, 961  
 Dicumarol induced hypoprothrombinemia in rats, effect of vitamin K on, 1431, 1651
- Diet, low fat, effect of choline, methionine, and, on life expectancy of patients with cirrhosis of liver, 1123  
 rice, dangers from use of, 1468  
 sodium, the minimal, its effect upon blood pressure of ambulatory hypertensive subjects, 1482
- Dietary influences in experimental anuria, 1489
- Diethylstilbestrol, effect of, on blood lipids and development of atherosclerosis in chickens on a normal and low fat diet, 733
- Diets, synthetic, 1059
- Digitalis (cedilamid) and quinidine, experimental studies on combined action of, in cats, 1493  
 effect of, on coagulability of blood, 1653
- Dihydroergocornine, effects of, on peripheral circulation in man, 1497
- Diphtheria and tetanus antitoxin, purification of, by use of pepsin, 757
- Disease, toxoplasma neutralizing antibodies in, 87
- Diseases, thymol turbidity test in various, 565
- Dog, heart, excitability and conduction velocity in, action of several cardiac glycosides on, 1491
- Dogs, anesthetized, tidal volume recorder for, 784  
 crushing of muscle in, bacteria induced shock resulting from, 502  
 intravenous injection of fat emulsion into, experimental studies, 689
- Duodenal ulcer, comparison of twelve hour nocturnal gastric secretion in uncomplicated, before and after healing, 1623
- Dwarfism, pituitary, anterior pituitary extract in treatment of, evaluation of, 1615
- E
- Edema in nephrosis, relief of, by plasma albumin and gelatin infusions, 1491  
 of various origins, electrolyte partition in patients with, 1534, 1545
- Egg albumin, increase of complement units by, 239
- Electric resistance of human blood, measurement of, use in coagulation studies and cell volume determinations, 1110
- Electrocardiogram, alterations of, by tilting, 951  
 effects of dicumarol on, 972  
 T wave of, factors influencing, experimental study employing intracavitary and epicardial leads, 1498
- Electrocardiographic and pathologic findings in infarction of interventricular septum, correlation of, 1456  
 leads, multiple and special, devices for rapid recording of, 118
- Electrolyte partition in patients with edema of various origins, 1534, 1545
- Electrophoretic study of blood serum from lymphogranulomatous patients, 624
- Emetine therapy in presence of heart disease, 1457
- Endamoeba histolytica, influence of oxyquinoline drugs on, as measured in blood iodine levels of man, 1632
- Endocrine imbalance, premature calcification of costal cartilages associated with, 1621
- Endometrium, beta glucuronidase activity of human, 1619
- Enterogastrone concentrates, effect of, on gastric secretion, 1622
- Erythroblastosis fetalis, conglutination test in, 181  
 in newborn, maternal isoimmunization without evidence of clinical, 526
- Erythrocyte counts, method of frequent estimations of circulating plasma volume with, 1650
- Erythrocytes in urinary sediment, identification and normal limits, 875  
 use of preserved, for detection and identification of Rh antibodies, 1177
- Estrogens, urinary, hydrolysis of conjugated, comparison of methods used for, 207
- Ethanol, microquantities of, in blood and other biologic fluids, colorimetric method for determination of, 241
- Ethylene glycol poisoning, clinical and pathologic study, 573
- Evelyn macrocolorimeter, a microadapter for, 1594
- Exophthalmic goiter, radioiodine tracers in, effect of varying dose of iodine on behavior of, 1615
- Extremities, blood flow in human, compensating plethysmograph for, 101
- F
- Familial hypoprothrombinemia, 542
- Fat emulsions for intravenous nutrition, 17a  
 intravenous injection of, into dogs, experimental studies on, 689  
 soybean phosphatides as emulsifiers for, 163  
 tolerance of dogs to, 143  
 metabolic studies on puppies infused with, 1503  
 preparation of, for intravenous nutrition improved techniques for, 153  
 in blood, turbidimetric determination of in fused, after intravenous administration of fat emulsions, 175
- Fever, Haverhill, bacteriologic diagnosis, 1605
- Fibrotic reactions, physical mechanism of, 1454
- Filariasis, chemotherapy of, 216
- Flocculation tests, combination of, in differential diagnosis of jaundice, 1630
- Folic acid antagonist treatment of chronic leucemia with, 1643  
 metabolism studies, 1271  
 treatment of acute leucemias with analogues of, 1641
- Food allergy, acute torticollis due to, 1614

4 Amino pteroylglutamic acid treatment of acute leucemia with 1642  
 Frozen sections preparation of, for use in chemosurgical technique for microscopically controlled excision of cancer, 392

## G

Gantrosan and penicillin prevention of bacteremia following tooth extraction with 1461  
 Gastric acidity response during intravenous administration of protein hydrolyates 1627  
   secretion effect of enterogastrone concentrates on 1622  
   twelve hour nocturnal, comparison of in uncomplicated duodenal ulcer before and after healing 1623  
   secretory response to histamine incidence of symptoms and in patients with and without chronic gastritis 1624  
 Globin and porphyrin metabolism made with C<sup>14</sup> and N<sup>15</sup> 1478  
 Glomerular filtration and effective renal plasma flow effects of cold pressor test on 430  
 Glucose tubular reabsorption of in diabetic patients effect of insulin on 1616  
 Glycine administered by vein efficacy and safety of 1555  
 Glycosuria simulated by the administration of ascorbic acid occurrence and differentiation 1471  
 Gout exophthalmic, radioiodine tracers in effect of varying dose of iodine on behavior of 1615  
 Gold compounds in treatment of arthritis metabolism toxicity and manner of action of 1381  
   distribution of in animal body in relation to arthritis 1133  
   toxicity in rats effect of BAL on 1024  
 Gout role of adrenal cortex in 1473

## H

Haverhill fever bacteriologic diagnosis 1608  
 Hayem's solution for red blood counts methods to increase accuracy in use of 1180  
   serum flocculation reaction with routine use of 1629  
 Heart disease congenital intravenous catheterization of heart in diagnosis of 1489  
   emetine therapy in presence of 1457  
   intravenous catheterization of in diagnosis of congenital heart disease 1489  
   sensitivity of to acetylcholine effects of acute hypoxia on 1495  
 Hemagglutinin test cold by a slide method 1447  
 Hematopoietic tissues radioactive (As <sup>63</sup>) in treatment of leucemia and allied disorders of 1649  
 Hemoglobin determination of as pyridine hemochromogen 1638  
   estimation crystalline hemin solutions as permanent standards for 783  
   oxygen saturation of recording of by a whole blood oximeter 1637

Hemolytic diseases plasma catalase in 487  
   transfusion reactions intragroup incompatibility with respect to H<sub>r</sub> blood factors as a cause of minor 98a  
 Hemophilia current theories and successful management in traumatic and surgical crises 708  
 Hemosiderin sternal marrow 81  
 Heparin and dicumarol comparative action of on the in vivo clot experimental study of 721  
   commercial effect of on platelet count, 1410  
   effect of on platelets in vivo 1424  
   use of intramuscular for anticoagulant therapy 1653  
 Hepatic cirrhosis needle biopsy of liver in experiences with 1627  
   qualitative and quantitative definition of cirrhosis and anions in 1545  
   dysfunction in infectious mononucleosis review of literature 1258  
 Hepatitis infectious inadvertently transmitted with therapeutic malaria 998  
   urine in acute antidiuretic activity of 1470  
 Heterophile antibodies demonstration of in cerebrospinal fluid in infectious mononucleosis 1204  
 Hetrazan pharmacodynamic properties of 216  
 Histamine administration during pregnancy clinical observations on 1601  
   antagonists 319  
   gastric secretory response to incidence of symptoms and in patients with and without chronic gastritis 1624  
   test for presence of hypertensive diencephalic syndrome using, 1470  
   treatment of retrobulbar neuritis 1611  
 Histoplasmosis complement fixation test for yeast phase antigens in 811 1207  
 Hodgkin's disease sternal marrow studies in 1640  
   syndrome studies in 1002  
 Hookworm need of differentiation of trichostrongylus infection in human beings from 869  
 Hormonal influence in experimental anuria, 1489  
 H<sub>r</sub> blood factors intragroup incompatibility with respect to as a cause of minor hemolytic transfusion reactions 985  
 Hydrolysis of conjugated urinary estrogens, comparison of methods used for 207  
 Hyperemia reactive failure of antihistaminic drugs to reduce 1609  
 Hyperglycemia effect of on clearances of insulin and paraaminohippuric acid 1617  
 Hypertension arterioles of skin in 1486  
   arteriovenous nicking in long term study of 1486  
   cholesterol tolerance tests in 1618  
   insulin tolerance in 1487  
   new method of studying 1483  
   pulmonary in chronic hypertrophic pulmonary emphysema 1453

- Hypertension—Cont'd  
thioevanate content of saliva in, before and after ingestion of drug, 772
- Hypertensive diencephalic syndrome, test for presence of, using histamine, 1470
- Hyperthyroidism, movement test in, electrocardiographic response to, 1500
- surgical management of, medical and metabolic factors in, 1618
- Hypertrophic pulmonary emphysema, pulmonary hypertension in chronic, 1453
- Hypoalbuminemia, frequency and significance of, studies in serum proteins, 1276
- Hypoferrinemia and other blood changes associated with injection of turpentine, influence of adrenalectomy and of adrenal cortical hormone on, 532
- Hypoprote thrombinemia, dicumol induced, in rats, effect of vitamin K on, 1431, 1651
- idiopathic (familial), 542
- Hypoparathyroidism, administration of penicillin and streptomycin by means of, 505
- Hypoxia, acute, effects of, on sensitivity of heart to acetylcholine, 1495

## I

- Idiopathic hypoprote thrombinemia, 542
- Ileus, pyloric balance in, 1625
- Infection of interventricular septum, electrocardiographic and pathological findings in, correlation of, 1456
- Infection, anemia of, 532
- response, nutritional status and, 47
- suppurative, in man, studies on prolonged, 1607
- Infectious mononucleosis, hepatic dysfunction in review of literature, 1258
- heterophile antibodies in cerebrospinal fluid in, 1204
- Influenza A viruses, antigenic differences among including serologic response of patients, 1212
- and pneumonia, 32
- serologic studies of, during and after outbreak of influenza B, 32
- in Boston, serologic studies of, during winter of 1945-1946, 15
- vaccination against, results of, during the epidemic of 1947, 789
- viruses, isolation and identification of, during epidemic of December, 1945, 309
- Insulin, effect of, on tubular reabsorption of glucose in diabetic patients, 1616
- tolerance in hypertension, 1487
- Interventricular septum infection of electrocardiographic and pathological findings in correlation of, 1456
- Intragroup incompatibility with respect to Hr blood factors as cause of minor hemolytic transfusion reactions, 985
- Inulin and para-aminohippuric acid, effect of hyperglycemia on clearances of, 1617
- Iodine, effect of varying dose of, on behavior of radioiodine tracers in exophthalmic goiter, 1615

- Iron stores in man, determination of available, method for, 81
- Isoagglutinin titers, anti A and anti B, in Rh immunized pregnant women, 1089

- Isoimmunization, maternal, without evidence of clinical erythroblastosis fetalis in newborn, 526

## J

- Juandice, required hemolytic, by Coombs serum, spleen as source of substance causing agglutination of red blood cells in, 1197
- differential diagnosis of, combination flocculation tests in, 1630
- Jet injection, administration of penicillin and streptomycin by means of, 505
- JSB strain, modified method of preparing, 777

## K

- Kahn test, antigen for, standardization of, 1224
- Kidney, a resin artificial, 841
- extracts, antibodies against renin and "assumed pressor principle" produced by, 1484

## L

- Labile factor of prothrombin complex, concentration of, in human, dog and rabbit blood, significance in determination of prothrombin activity, 819
- Laboratory methods, 96, 236, 357, 511, 644, 777, 975, 1037, 1169, 1311, 1440, 1588
- Lactate, blood and urine, influence of physical fitness and convalescence from major surgical procedures on the magnitude of the responses to exercise of, 1474
- Lactobacillus counts, correlation of, with extent of dental caries in an institutional population, 1614
- Lecithin, sensitivity of, variations in different lots of purified, 1220
- Letal procedures, effect of various, and thermal injury on capillaries, 497
- Leukemia, acute, induced by aminopterin, changes in blood and bone marrow in, 1451
- effect of urethane on, further studies on, 1647
- radiation (As<sup>90</sup>) in treatment of 1649
- treatment of acute, with aminopterin (4-aminopteroviglutamic acid), 1642
- with analogues of folic acid, 1641
- chronic with a folic acid antagonist, 1643
- Life span of sickle cell and the pathogenesis of sickle cell anemia, 975
- Ligation of pancreatic ducts, effect of and of pancreatectomy after duct ligation on serum lipase, 533
- Lipids, ingestion of various, influence of upon the thymol turbidity index, 1640
- Liver, alcoholic fatty, observations on with interval needle biopsy and liver function tests, 555

## Liver—Cont'd

- antipermeous anion factor of and pteroylglutamic acid for hemopoiesis in animals of both 164
- cirrhosis of blood and bone marrow in 1025
- life expectancy of patients with effect of choline methionine, and low fat diet on 1123
- dietary serum choline toxic determinations in 1475
- extract and vitamin B<sub>12</sub> effect of on mucous membrane lesions of microcytic anemia 1019
- function bromsulphalein test of importance of rate of dye removal in 445
- serum albumin globulin ratio cephalin cholesterol flocculation and thymol turbidity tests for a comparative study 462
- tests in observations on alcoholic fatty liver 555
- needle biopsy of in hepatic cirrhosis experienced with 162
- portal cirrhosis of chemical and functional study 1475
- structural and functional alteration of relation between 43
- lower nephron nephrosis management and clinical course of 1606
- lung action of pilocarpine on in normal and asthmatic subject 583
- lymph collection of from liver small intestine or thoracic duct of rat techniques for 1349
- lymphadenitis acute bacterial pathogenesis of 1435
- lymphoblastoma cutis and scleroderma paraneoplastic and therapy in 1462
- lymphogranulomatous patients electrophoretic study of blood serum from 624
- lymphoid cell extracts antibody content of determination of 1563
- lyophilized amino acid preparation a new determination of nitrogen balance index of in protein deficient patients 280

## M

- Macrocolorimeter Evelyn a microcolorimeter for 1594
- Microcytic anemia mucous membrane lesions of effect of liver extract and vitamin B on 1019
- Malaria sporezoite induced virus incidence of false positive tests for syphilis and 747
- therapeutic infectious hepatitis inadvertently transmitted with 98
- use of Iodoquin virus preserved by freezing in medium 159
- Malignant hypertensive persons effects of bacterial pyrogens on 1469
- Mammalian striated myofibril preparation and properties of 1612
- Mindclamine (methenamine mindlate) absorption distribution and renal excretion of 1304

- Measurement of surface temperature of body thermocouples for improved mounting for 1014
- Megalocyte and hemolytic syndrome of pernicious anemia life span of 1064
- Mercurial diuretic for subcutaneous injection experiences with 1635
- Mercuric bichloride for biologic studies factors in use of with especial reference to blood counts 1094
- Mescaline hallucinations effect of ucinato in 1297
- Metabolic studies on puppies infused with fat emulsions 150
- Metabolism globin and porphyrin made with C<sup>14</sup> and N<sup>15</sup> 1475
- protein dynamics of 257-60
- study folie acid 171
- Methenamine mandelate absorption distribution and renal excretion of 104
- Methionine choline and low fat diet effect of on life expectancy of patients with cirrhosis of liver 1123
- Methylis (B chloroethyl) amine hydrochloride clinical observations with 1648
- Micro and macroelectrophoretic analysis of human and rat serum a comparative study of 55
- Microcolorimeter for the Evelyn macrocolorimeter 1594
- Microbial animal protein factor concentrate a study of in pernicious anemia, 860
- Microcolorimetric determination of sodium in human biologic fluids 246
- Micrometer burette a Scholander of simple construction 1193
- Micromethod for determination of human albumin globulin and hemoglobin contents 1440
- of serum streptomycin levels 896
- Microorganisms pathogenic use of splenic infusion as a base for growing certain 1031
- Microtechniques for identification of cultures quick 1334-1338
- Molybdenum iron complex anemia of pregnancy treated with 1641
- Mononucleosis infectious hepatic dysfunction in review of literature 1258
- heterophile antibodies in cerebrospinal fluid in 1204
- thrombocytopenia secondary to 1252
- Mycetis following administration of neocarsinamine 1165
- Myocardial infarction dicumarol in experimental 981
- extremity and precordial leads in diagnosis of acute a comparative evaluation of 1500
- observations of 100 patients with who survived up to six years 1488
- Myofibrils mammalian striated preparation and properties of 1612

## N

- Needle biopsy of liver in hepatic cirrhosis experiences with 1627
- Necropsyphenamine myelitis following administration of 1165

- Neohetramine, a new antihistaminic agent, experimental and clinical studies, 565
- Nephron nephrosis, management and clinical course of lower, 1606  
nature of the altered renal function in lower, 1468
- Nephrosis, edema in, relief of, by plasma albumin and gelatin infusions, 1491
- Neuritis, retrobulbar, treatment with histamine, 1611
- Newborn, erythroblastosis fetalis in, maternal isoimmunization without evidence of clinical, 526
- Nicotinic acid amide, effect of, on experimental tuberculosis of white mice, 1249
- Nitrogen balance index, determination of, in a new lyophilized amino acid preparation in protein deficient patients, 280  
studies, comparative, of oral protein derivatives and natural protein in protein deficient patients, 1634  
mustard and X irradiation, effect of, on blood coagulation, 1566  
inhibitory effect of, on development of humoral antibodies, cutaneous hypersensitiveness and vascular lesions in rabbits following injections of horse serum, 1463  
therapy, 1002
- Normoblasts in peripheral blood, appearance of, in patients with pulmonary embolism, 1502
- Nutrition and experimental diabetes, 1161  
effect on tumor response in Rous chicken sarcoma, 1139  
intravenous, fat emulsions for, 175  
improved techniques for preparation of, 153  
parenteral, 143, 153, 163, 175, 1503
- Nutritional status and infection response, 47
- O
- 1 Diethylcarbamiyl 4 methylpiperazine hydrochloride, pharmacodynamic properties of, 216
- Ossilograph, direct writing, for recording data obtained by method of cardiac catheterization, 1499
- Oximeter, whole blood, recording of oxygen saturation of hemoglobin by, 1637
- Oxyhemograph, studies on cardiorespiratory function with, 1454
- Oxyquinoline drugs, influence of, on growth of *Endamoeba histolytica*, as measured in blood iodine levels of man, 1632
- P
- Palpitation, 1487
- Pancreatic calcification, diffuse, with diabetes mellitus, 1619  
ducts, effect of ligation of, and of pancreatotomy after duct ligation on serum lipase, 833  
function, studies on, 1047
- Para aminobenzoic acid, effect of, on metabolism and excretion of salicylate, 1393  
therapy in scleroderma and lymphoblastoma cutis, 1462
- Parenteral nutrition, 143, 153, 163, 175, 1503
- Paroxysmal tachycardia, low nodal, benefited by cardiac sympathectomy, 1493
- Pathogenesis of acute bacterial lymphadenitis, 1458
- Pathologic specimens, imbedding of, in transparent plastic, 1311
- Penicillin and gantrosan, prevention of bacteremia following tooth extraction with, 1461  
and streptomycin, administration of, by means of hypodermic apparatus (jet injection), absorption, toxicity and stability, 805  
in isolation of poliomyelitis virus from fecal specimens, 1402  
in treatment of actinomycosis, 521  
levels, high blood, achieved with caronamide, role of, in penetrating the blood brain barrier, 933  
plasma protein fractions in action and transport of, 1608  
procaine, therapeutic efficiency and comparative study of absorption, 1232  
rectal absorption of, 135  
response of bacterial populations to the action of, 417
- Pepsin, purification of diphtheria and tetanus antitoxin by use of, 757
- Peptic ulcer, bleeding, 1624
- Peripheral circulation in man, effects of dihydroergocornine on, 1497
- Pernicious anemia, activity of microbial animal protein factor concentrates in, 860  
cholinesterase activity of blood in, 149  
life span of the megalocyte and the hemolytic syndrome of, 1068
- Pharmacology of heptazan, 216
- Phenol production, effect of phthalylsulfathiazole and streptomycin on, 840  
retention, relation of, to uremia, 845
- Phospholipids, high vitamin A intake and blood levels of, 67
- Phthalylsulfathiazole and streptomycin, effect of, on phenol production, 840
- Pilocarpin, action of, on lungs in normal and asthmatic subjects, 585
- Pituitary adrenocorticotrophic hormone, production of temporary diabetes mellitus with, 651  
dwarfism, anterior pituitary extract in treatment of, 1615
- Plasma, albumin, and globulin in determination of, a rapid chemical method for the accurate, 367  
catalase in hemolytic diseases, 487  
in normal adults, method for determination of, and values obtained, 451  
concentrations of caronamide, 297  
corticosteroids in, methods for chemical determination of, 1326  
defect in patients whose bleeding is temporarily controlled by protamine



## Plasma—Cont'd

- sulfate or toluidine blue 140
- protein fractions role of in action and transport of penicillin 1408
- of man effect of oral administration of casein hydrolysate on total circulating 453
- studies in children with rheumatic fever 1490
- vitamin A alcohol influence of endogenous and nutritional factors upon 1631
- volume circulating method of frequent estimations of with erythrocyte counts 1650
- Plasmodium vivax* preserved by freezing use of in inducing malaria 1579
- Plastic transparent imbedding of pathological specimens in 1311
- Platelet count effect of commercial heparin on 1410
- Platelets in vivo effect of heparin on 1424
- Plethysmograph a compensating for measuring blood flow in human extremities 101
- Pneumococcus hyaluronidase* in serum of normal human beings and patients with *pneumococcus pneumoniae* antihyaluronidase antagonistic to 919
- Pneumonia and influenza 3
- serologic studies of after outbreak of influenza B 32
- puerperae pneumococcus* rise of titer in 919
- Poisoning ethylene glycol clinical and pathologic study 573
- Poisons common steam volatile organic systematic qualitative analysis of biologic materials for 371
- Poliovirus* virus isolation of from fecal specimens use of penicillin and streptomycin in 140
- Polyethylene tubing venous catheterization with 159
- Porphobilinogen tests on 1000 patients in search for false positive reactions 1254
- Portal cirrhosis of liver clinical and functional study 1470
- Preprecipitation reaction determination of human albumin by means of chemical method for 1052
- Pregnancy anemia of treated with molybdenum iron complex 1641
- blood pressure maintenance in normal and toxemic using tetraethylammonium chloride evaluation of neurogenic and humoral factors in 1466
- histamine administration during clinical observations on 1601
- Pregnandiol colorimetric method for determination of a new 251
- in urine quantitative colorimetric estimation of simplified technique for 356
- Pregnant women Rh immunized anti A and anti B isoagglutinin titers in 1089

## Pregnant women—Cont'd

- urinary volume of effect of sedatives on 1460
- Incident a address Central Society for Clinical Research October 29 1948 1458
- Intracerebral elevations mechanism of sustained effects of adrenalin in normal and hypertensive patients in relation to 332
- Procaine penicillin therapeutic efficiency and a comparative study of absorption 1232
- Propylthiouracil and cholesterol, effect of feeding on blood cholesterol and arterial intima in rat 1029
- Protamine rutin and benadryl, influence of on experimental purpura in the guinea pig 1651
- ultra bleeding temporarily controlled by plasma defect in 1480
- Protein hydrolysates gastric acidity response during intravenous administration of 1627
- metabolism dynamics of 257 269
- requirements of adult dogs studies on the minimum 482
- Infantile patients comparative nitrogen balance studies of oral protein derivatives and natural protein in 1634
- Prothrombin activity concentration of labile factor of prothrombin complex in human dog and rabbit blood significance of in determination of 819
- Pseudohypoparathyroidism 1620
- Pittuitary virus acquired resistance of to sulfadiazine and effects of chemical antagonists on sulfonamide activity 1241
- Psychoneuroses premature calcification of costal cartilages associated with 1621
- Psychosomatic problems in a private practice 1621
- Pteroylglutamic acid and pteroylglutamic acid intravenous administration of in folic acid metabolism studies 1271
- antagonists of use of in controlling Rous chicken sarcoma 1144
- deficiency in swine effect of treatment with pteroylglutamic acid liver extract and protein 297
- for hematopoiesis and antipernicious anemia factor of liver essentiality of both 1647
- Pulmonary carcinoma sputum cell study for as routine laboratory test 1558
- embolism peripheral blood of patients with appearance of normoblasts in 1502
- hypertension in chronic hypertrophic pulmonary emphysema 1453
- Purpura influence of rutin benadryl and protamine on experimental in the guinea pig 1651

# Purpura—Cont'd

- thrombopenic, failure of direct blood transfusion to raise the platelet level, 1077
- Pyloric balance in ileus, 1625
- Pyribenzamine and benadryl, comparison of toxic manifestations produced by, 595
- Pyridine hemochromogen, determination of hemoglobin as, 1638

# Q

- Quantitative complement fixation test for syphilis, method for determination of titers between 10 and 100 in, 383
- ischemia in limbs of animals, apparatus for producing, 1450

# R

- Radioactive isotopes from biologic tissue, recovery and estimation of, 1169
- Radioimmunity induced is stable, tissue distribution of, 635
- Radioactive ( $^{137}\text{Cs}$ ) in treatment of leukemia and allied disorders of the hematopoietic tissues, 1649
- Radiiodine tracers in exophthalmic goiter, effect of varying dose of iodine on the behavior of, 1615
- Reactive hyperemia, failure of antihistaminic drugs to reduce, 1609
- Recording electrocardiographic leads, multiple and special, devices for rapid, 118
- Rectal absorption of penicillin, 135
- Red blood cells, destruction of, studies on, 1197
  - counts, Hayem's solution for, methods to increase accuracy in use of, 1180
- Renal function, altered, nature of, in lower nephron nephrosis, 1468
  - hypertension, effect of desoxycorticosterone acetate and propylene glycol in experimental, 1483
- Resin utilized kidney 841
- Respiratory and circulatory systems, relation of tobacco smoking to diseases of, 1497
- Retinal arteriovenous nicking, 1486
- Retinobulbar neuritis, treatment with histamine 1611
- Rh antibodies, detection and identification of, use of preserved erythrocytes for, 1177
  - studies on, 1353, 1361, 1369
- antisera, analysis of a zone phenomenon in, by splitting the serum into two fractions by means of dialysis, 1353
- Rh immunized pregnant women, anti A and anti B isogglutinin titers in, 1089
- Rheumatic fever, plasma protein studies in children with, 1490
  - renal excretion of salicylate during treatment of acute, 1636
- Rice diet, dangers from use of, 1468

- Rous chicken sarcoma, effect of nutrition on tumor response in, 1139
  - use of antagonists of pteroylglutamic acid in controlling, 1144
- Rutin, benadryl, and protamine, influence of on experimental purpura in guinea pig, 1651
  - effect of, upon resorptive toxicity of certain drugs, 1464

# S

- Salicylate, metabolism and excretion of, effect of para aminobenzoic acid on, 1393
  - renal excretion of, during treatment of acute rheumatic fever, 1636
- Saliva, thiocyanate content of, in normal and hypertensive subjects before and after ingestion of the drug, 772
- Salmonella bleedum, occurrence of, in Louisiana, 1155
- Sarcoma, Rous chicken, effect of antagonists of pteroylglutamic acid in controlling, 1144
  - of nutrition on tumor response in, 1139
- Scholander micrometer burette of simple construction, 1193
- Scleroderma and lymphoblastoma cutis, para aminobenzoic acid therapy in, 1462
- Screening method for blood glucose, 236
- Screw syringe, 1603
- Sedative drug, a new (3,3 diethyl 2,4 dioxypiperidine) evaluation of, 602
- Sedatives, effect of, upon urinary volume of pregnant women, 1465
- Sedimentation rate technique, simplified, with combined chart and correction nomogram, 511
- Sensitivity, cotton bean, 1613
  - of lecithin, variations in different lots of purified, 1220
- Serologic studies, influenza and pneumomun during and after outbreak of influenza B, 32
  - in Boston during winter of 1945-1946, 15
- Serotonin crystallization of, 1606
- Serum, albumin and globulin in, determination of, a rapid chemical method for the accurate, 367
  - albumin globulin ratio, cephalin cholesterol flocculation test, and thymol turbidity test for liver function a comparative study, 662
  - antitubercular cytotoxic, in arthritis, 765
  - failure of, 1289
- calcium determination, rapid method for, 906
- cholesterase determinations in liver disease, 1475
- coagulation reaction, Weltmann further studies on, 618
- dilution turbidity test in, in comparison with thymol turbidity and cephalin cholesterol flocculation test, 672

## Serum—Cont d

- disease in rabbits, effect of sodium chloride upon 936
- flocculation reaction with Hayem's solution routine use of 1629
- human and rat micro and macroelectrophoretic analysis of comparative study, 75
- lipase, effect of ligation of pancreatic ducts and of pancreatectomy after duct ligation on 833
- simplified method for determination of using aqueous tributyrin as substrate 1047
- proteins of blood dyscrasias electrophoretic analysis of 15-3
- studies in 36, 126
- streptomycin levels micromethod for determination of 886
- Shock bacteria induced resulting from eruptions of mule in dogs mechanism of 602
- Sickle cell anemia pathogenesis of and the life span of the sickle cell 975
- Sickling of red blood cell simple and rapid method for demonstrating use of reducing agents 1082
- Skin arterioles of in essential hypertension 1486
- Slide method of cold hemagglutinin test 1447
- Sodium diet the minimal its effect on blood pressure of ambulatory hypertensive subjects 148
- in human biologic fluids microcolorimetric determination of 246
- salicylate effect of upon sodium chloride in rabbits 936
- Soybean phosphatides as emulsifiers for intravenous fat emulsions, 163
- Spectrophotometric determination of blood pH 881
- of urinary bilirubin 1189
- Splanchnectomy effect of on blood pressure and on cardiac and renal function 1455
- Splenic infusion, use of as base for growing certain pathogenic microorganisms 1037
- Sputum cell study for pulmonary carcinoma as routine laboratory test 1583
- Stain JSB a modified method of preparing 777
- Sternal marrow hemosiderin 81
- tubes in Hodgkin's disease 1640
- Steroid diabetes associated with Cushing's syndrome and excretion of 17-hydroxycorticosterone (compound F) in urine metabolic studies 1442
- Stomach acidity variations in stimulated and nonstimulated diagnostic and therapeutic implications of 1646
- in cancer of cytologic diagnosis of 1639
- Stools streptomycin resistant bacteria in, development of in patients treated for tuberculosis 1460
- Streptomycin and penicillin administration of by means of hypodermic apparatus (jet injection) absorption toxicity and stability 805

## Streptomycin and penicillin—Cont d

- in isolation of poliomyelitis virus from fecal specimens 1402
- and phthalylsulfathiazole, effect of, on phenol production 845
- experimental therapy of generalized torulosis in rats with 1159
- urum levels, micromethod for determination of 886
- therapy in United States Army hospitals 1
- Streptomycin resistant bacteria, development of in stools of patients treated for tuberculosis 1460
- Subtilin topical application of, to tuberculous lesions in man 199
- Succinate effect of in mercurine hallucinations 1297
- Sulfadiazine, acquired resistance of psittacosis virus to and effects of chemical antagonists on sulfonamide activity 1241
- Suppurative infection prolonged 1607
- Surface temperature of body thermocouples for measurement of, improved mounting for 1044
- Synthetic diets 1059
- Syphilis, false positive tests for incidence of in sporozoite induced vivax malaria 747
- in acute hemolytic anemia transient positive Wassermann test for 753
- quantitative complement fixation test for method for determination of titers between 10 and 100 in 383
- transient positive Wassermann test for, in acute hemolytic anemia 753
- Syringe screw 1603

## T

- T wave of electrocardiogram factors influencing experimental study employing intracavitary and epicardial leads 1498
- Test, conglutination in erythroblastosis fetalis 191
- Titanium antitoxin purification of by use of pep in 157
- Tetraethylammonium chloride blood pressure maintenance in normal and toxemic pregnancy using evaluation of neurogenic and humoral factors in 1466
- effects of on blood flow in extremities of man 743
- Therapeutic malaria, infectious hepatitis in, adversely transmitted with 998
- Thermal trauma delayed death following, mechanism of 506
- Thermocouples for measurement of surface temperature of body improved mounting for 1044
- Thiocyanate content of saliva in normal and hypertensive subjects before and after ingestion of the drug 742
- Thionamrin a mercurial diuretic for subcutaneous injection experience with 1675
- 3,4-Dichloro-4-dioxopiperidine a new editive drug, evaluation of 602

- Thrombocytopenia secondary to infectious mononucleosis, 1652
- Thrombocytopenic purpura, coagulation defect in, 1479
- Thrombopenic purpura, failure of direct blood transfusion to raise the platelet level, 1077
- Thymol turbidity and cephalin cholesterol flocculation tests in serum, a dilution turbidity test in comparison with, 672
- influence of ingestion of various lipids upon, 1630
- test in various diseases, 565
- serum albumin globulin ratio, and cephalin cholesterol flocculation test for liver function, a comparative study, 662
- Tidal volume recorder for anesthetized dogs, 784
- Tissue distribution of radioantimony inhaled as stibine, 635
- Tobacco smoking, relation to diseases of respiratory and circulatory systems, 1497
- Toluidine blue, bleeding temporarily controlled by, plasma defect in, 1480
- Torticollis, acute, due to food allergy, 1614
- Torulosis generalized, experimental therapy of, in rats, with streptomycin, 1159
- Toxoplasma neutralizing antibodies, occurrence of, in various disease conditions, 87
- Trichostrongylus infection in human beings, need of differentiation from hook worm, 869
- Tripelennamine and beta dimethylaminoethyl benzhydryl ether hydrochloride, comparison of toxic manifestations produced by, 595
- Tuberculosis, experimental, effect of nicotinic acid amide on, in white mice, 1249
- Tuberculous lesions, topical application of subtilin to, 799
- Turpentine, blood changes associated with injection of, influence of adrenal ectomy and of adrenal cortical hormone on, 532
- 2 [ $\alpha$  (2 dimethylaminoethoxy)  $\alpha$  methylbenzyl] pyridine succinate, clinical and experimental results of, 319
- pharmacologic properties of, 325
- 2,3 Dithiopropanol, effect of, on gold toxicity in rats, 1024
- Typhoid carrier state, treatment of, 1150
- U
- Ulcerative colitis, duodenal substance in chronic, clinical evaluation of, 1633
- United States Army hospitals, streptomycin therapy in, 1
- Urate metabolism, role of adrenal cortex in, 1473
- U.      on of phenol retention to, 845
- U      effect of, on leucemia, further studies on, 1647
- Urinary bilirubin, spectrophotometric for determination of, 1189
- estrogens, hydrolysis of conjugated, comparison of methods used for, recovery from caronamide, 297
- sediment, erythrocytes in, identification and normal limits, 875
- volume of pregnant women, effect of lives on, 1465
- Urine, bilirubin in, quantitative determination of, method for, 890
- corticosteroids in, methods for determination of, 1326
- excretion of 17 hydroxycorticosteroids (compound F) in, steroid diabetes associated with Cushing's syndrome and, metabolic studies, 1472
- in acute hepatitis, antidiuretic activity of, 1476
- pregnandiol in, quantitative colorimetric estimation of, simplified technique for, 356
- V
- Vaccination against influenza, results of, during epidemic of 1947, 789
- Venous catheterization with polyethylene tubing, 1597
- Ventricular irregularities induced by sympathetic adrenal discharge and chloroform, 1438
- Vitamin A intake, high, and blood levels of cholesterol, phospholipids, carotene, and Vitamins C, A, and E, 67
- B<sub>12</sub> and liver extract, effect of, on mucous membrane lesions of macrocytic anemia, 1019
- effect of, on hematopoietic and nervous systems in Addisonian pernicious anemia, 1646
- in pernicious anemia and puerperal macrocytic anemia, 1477
- K, effect of, on dicumarol induced hypoprothrombinemia in rats, 1431, 1651
- Volatile reducing substances (alcohol or ether) in blood and gases, determination of, using barium diphenylamine sulfonate as an indicator for chromic acid titration, 126
- W
- Wassermann test, transient positive, for syphilis in acute hemolytic anemia, 753
- Water by vein, effect of, on the more severe complications of cardiovascular renal disease, 1467
- Weltmann serum coagulation reaction, further studies on, 618
- Whole blood oximeter, recording of oxygen saturation of hemoglobin by, 1637
- Y
- Yeast phase antigens in complement fixation test for histoplasmosis, 511, 1207

